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Date May 8, 2000.
Abstract

MHC class I molecules contain within their cytoplasmic domains a tyrosine and two or three serines that demonstrate a remarkable level of conservation between all mammals and extending to species as evolutionarily distant as the shark and zebrafish. In order to explore the role of these amino acids both in vitro and in vivo, cytoplasmic mutants of the murine H-2 K\(^b\) gene were constructed and expressed in murine L cell fibroblasts and transgenic mice.

In L cell fibroblast transfectants, K\(^b\) mutant molecules reached higher cell surface levels than K\(^b\) wild-type (K\(^b\)WT) molecules and demonstrated a higher capacity for binding exogenously-added peptides. While cytoplasmic mutations had no effect on \(\beta_2\)-microglobulin binding nor K\(^b\) transport rates to the cell surface, they caused a delay in the ability of the molecules to present an immunodominant vesicular stomatitis virus (VSV) epitope following viral infection.

In the transgenic mice, FACScan analysis of peripheral blood leukocytes and splenocytes revealed that cytoplasmic mutant K\(^b\) molecules reached significantly higher surface levels compared to wild-type K\(^b\) and interfered less with endogenous class I maturation and surface expression. Constitutive endocytosis of class I molecules from the surface of activated T cells was also shown to be significantly impaired by cytoplasmic point mutations.

Functional studies on antigen presentation in the transgenic mice revealed that mice containing a point mutation to the conserved tyrosine were severely compromised in their ability to generate a K\(^b\)-restricted CTL response to VSV infection compared to K\(^b\)WT-expressing and C57/BL control mice. Mice containing mutations to the conserved serines by contrast, demonstrated a more vigorous VSV CTL response compared to K\(^b\)WT-expressing transgenic animals.

The results demonstrate that the conserved tyrosine and serine residues are important for regulating lymphocyte and fibroblast MHC class I cell surface expression both in vitro and in vivo, and provide evidence of an inducible tyrosine-based endosomal targeting motif in the cytoplasmic tail of class I molecules. The functional studies suggest that the tyrosine motif in particular may be important for the generation of class I-restricted CTL responses in vivo.
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List of Abbreviations

$^{35}$S  
Sulphur-35

$^{51}$Cr  
Chromium-51

$^{125}$I  
Iodine-125

AFU  
arbitrary fluorescence unit

APC  
antigen presenting cell

ATP  
adenosine triphosphate

$\beta_2m$  
beta-2 microglobulin

Ca$^{2+}$  
calium

CCV  
cloathrin-coated vesicle

Con A  
Concanavalin A

CTL  
cytotoxic T lymphocyte

DC  
dendritic cell

DMEM  
Dubleco’s Modified Eagle Medium

DNA  
deoxyribonucleic acid

E:T  
effector:target

EGF  
epidermal growth factor

Endo H  
endoglycosidase H

EPLC  
exogenous peptide-loading compartment

ER  
endoplasmic reticulum

EtBr  
ethidium bromide

FACScan  
fluorescence-activated cell scan

FACSort  
fluorescence-activated cell sorter

FBS  
fetal bovine serum

FITC  
fluorescein isothiocyanate

GPI  
glycosylphosphatidylinositol

H-2  
Histocompatibility-2

HC  
heavy chain

HIV  
human immunodeficiency virus

HLA  
human leukocyte antigens

Ig  
immunoglobulin

Ii  
invariant chain

IL-2  
interleukin-2

IP  
immunoprecipitated

ITAM  
Immunoreceptor family tyrosine-based activation motif

K$^b$WT  
H-2 K$^b$ molecule, wild-type

kD  
kiloDalton

KIR  
killer inhibitory receptor

LC  
Langerhans cells

LMP  
low molecular weight proteosome protein

MHC  
Major Histocompatibility Complex

MLR  
mixed leukocyte reaction
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Definition</th>
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<tr>
<td>MOI</td>
<td>multiplicity of infection</td>
</tr>
<tr>
<td>MPR</td>
<td>mannose-6-phosphate receptor</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>MW</td>
<td>molecular weight</td>
</tr>
<tr>
<td>NH₄Ac</td>
<td>ammonium acetate</td>
</tr>
<tr>
<td>NK</td>
<td>natural killer cell</td>
</tr>
<tr>
<td>OVA</td>
<td>ovalbumin</td>
</tr>
<tr>
<td>p.i.</td>
<td>post-infection</td>
</tr>
<tr>
<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PBL</td>
<td>peripheral blood leukocyte</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PE</td>
<td>phycoerethrin</td>
</tr>
<tr>
<td>PKA</td>
<td>protein kinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>RE</td>
<td>restriction enzyme</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RPM</td>
<td>rotations per minute</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium lauryl sulphate</td>
</tr>
<tr>
<td>Ser</td>
<td>serine</td>
</tr>
<tr>
<td>TAP</td>
<td>Transporter Associated with Antigen Processing</td>
</tr>
<tr>
<td>TCA</td>
<td>trichloroacetic acid</td>
</tr>
<tr>
<td>TcR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TE</td>
<td>Tris-EDTA</td>
</tr>
<tr>
<td>Thr</td>
<td>threonine</td>
</tr>
<tr>
<td>TNF</td>
<td>tumour necrosis factor</td>
</tr>
<tr>
<td>Tyr</td>
<td>tyrosine</td>
</tr>
<tr>
<td>VSV</td>
<td>vesicular stomatitis virus</td>
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This thesis is dedicated to my partner Debbie Lynn Chow, my father Gerald Joseph Lizée, my mother Jane Frances Mills, and my stepfather Brian John Mills. Your unwavering love, support and sacrifices have made this long journey possible and for this I will always be exceedingly grateful.
Chapter 1 - General Introduction

1.1 The Major Histocompatibility Complex

The major histocompatibility complex (MHC) is a genomic region comprised of several genes encoding proteins with primarily immunological functions. These include MHC class I and class II molecules, antigen processing components (TAP and LMP), complement proteins, and cytokines such as tumor necrosis factor (TNF) and lymphotoxin (LT). The MHC also contains many other open reading frames that encode proteins of as yet unknown function, in addition to several pseudogenes. In humans, the MHC is located on the short arm of chromosome 6, whereas in mice it is found on chromosome 17. The human MHC occupies a large segment of DNA, extending some 3500 kilobases (kb). While the murine MHC is somewhat smaller, it shows very similar genomic organization with the exception of one major rearrangement (see Figure 1-1). Many of the genes in the MHC complex are related members of the Ig gene superfamily and appear to have arisen by gene duplication. Some of the genes are highly polymorphic, a factor that contributes significantly to the wide diversity of potential immune responses inherent within species populations.

Since it is often best to start things at the beginning, this thesis will begin with an examination of the MHC from a historical perspective…
1.1.1 A Brief History of the MHC

1.1.1.1 Mouse Studies

The MHC was originally discovered in the 1940s as a result of tissue transplantation experiments involving inbred strains of mice. Using a classical genetic approach, congenic mouse strains were bred that differed only in the genes responsible for causing tissue graft rejection. These studies indicated that although several genes could contribute to rejection, a single genetic region was responsible for most rejection phenomena (1, 2). This region, called Histocompatibility-2 (H-2), contained two genetic loci, H-2K and H-2D, which were found to be the most important loci for controlling graft rejection in mice. The K gene was first discovered in a mouse strain whose MHC had been designated “k”, and the D gene was first discovered in a mouse strain whose MHC had been designated “d”. The nomenclature that followed designated that the allele of the K gene found in a strain with the k-type MHC is called K\textsuperscript{k}, whereas the allele of the K gene in a strain of d-type MHC is referred to as K\textsuperscript{d}. A third locus similar to K and D was subsequently discovered and designated L (3).

Almost twenty years after the discovery of these loci, the importance of the MHC in physiological immune responses was demonstrated by the discovery that the ability of inbred strains of mice and guinea pigs to produce antibodies in response to immunization with simple polypeptide antigens was an autosomal dominant trait that mapped to the MHC region (4). The genes that controlled such immune responses were
Figure 1-1: Map of the major histocompatibility complex.

This map of the human MHC is simplified to exclude other Class I- and Class II-like genes, genes not of immunological interest, and numerous genes of unknown function. The mouse MHC is similarly organized but is smaller in size (~2000 kb) and contains a translocation of the K gene (homologous to HLA-A in humans) to a region located 5' of the Class II region. Figure taken from “Cellular and Molecular Immunology, 3rd ed.” by Abbas, Lichtman, and Pober 1997.
called immune response (Ir) genes and were assigned to a region within the MHC called “I”, which mapped to the region between the K and D genes. This region was further subdivided into I-A and I-E subregions on the basis of recombination events during the breeding between congenic strains. The I region was also found to code for certain cell surface antigens against which antibodies could be produced by inter-strain immunizations (3). These molecules are now known to be encoded by the I-A and I-E genes. The nomenclature for designating alleles parallels that for the K and D genes, such that an inbred H-2^k haplotype mouse strain expressing K^k and D^k alleles also expresses I-A^k and I-E^k alleles.

1.1.1.2 Human studies

The development of blood transfusion and organ transplantation as methods of treatment in clinical medicine provided the impetus for defining the human genes that control rejection phenomena. It was noted that patients who rejected kidneys or had transfusion reactions to white blood cells frequently produced circulating antibodies that reacted to antigens on the surface of white blood cells from the organ or blood donor (4). These so-called “alloantisera” (alio-: foreign) contained alloantibodies that proved to be instrumental in defining at least six polymorphic genetic loci which were clustered together in a single area of the human genome and which could be used to help predict graft rejection. Due to their high levels of expression in human leukocytes, these alloantigens were called human leukocyte antigens (HLAs). The first three genes were defined using serological approaches and were called HLA-A, HLA-B, and HLA-C. An
adjacent region, HLA-D, was originally discovered by the induction of T cell proliferation in mixed leukocyte reactions. The three genes that mapped to this region were serologically defined and designated HLA-DR, HLA-DQ, and HLA-DP.

The HLA region, now recognized to be the human MHC, is equivalent to the H-2 region of mice in that the various HLA and H-2 loci are structurally and functionally homologous. In particular, human HLA-A, -B, and -C resemble murine H-2 K, D, and L and are called MHC class I molecules. Likewise, human HLA-DP, -DQ, and -DR resemble murine I-A and I-E and are referred to as MHC class II molecules (5). Every vertebrate species thus far examined shares these homologous polymorphic genes and serologically definable protein products.

The studies of the mouse MHC were accomplished using a limited number of inbred and congenic strains. Therefore, only 10 to 15 alleles were defined at each locus of the murine MHC (6). The human serological studies, by contrast, were done on an outbred human population. This approach, combined with genetic and molecular analyses, has revealed hundreds of alleles at certain HLA loci, demonstrating a level of polymorphism that is unprecedented in human genetics (7). MHC genes have since proven to be by far the most polymorphic genes present in the genome of every species analyzed (8, 9). The significance of this polymorphism will become apparent when the structure and function of MHC molecules are discussed.
1.1.1.3 Nature of T cell Recognition

The mixed leukocyte reaction (MLR), an in vitro model for allograft rejection, proved to be an effective test for studying T lymphocyte recognition of foreign MHC molecules (10). It was concluded from MLR experiments that two distinct classes of T lymphocytes recognize and respond to different MHC gene products (11). CD4\(^+\) T cells, most of which are cytokine-producing helper cells, are specific for class II molecules. CD8\(^+\) T cells, most of which are cytolytic T lymphocytes (CTLs), are specific for class I molecules. However, it was not until the structures of the MHC molecules were uncovered in greater detail that the significance of these MHC recognition specificities of CD4\(^+\) and CD8\(^+\) T cells became apparent. It was realized that these specificities applied not only to the recognition of foreign MHC but, more significantly, to recognition of other foreign protein antigens in the context of self MHC (12, 13). By specifically binding fragments of these foreign protein antigens and displaying them on the cell surface, MHC molecules play a fundamental role in the immune system’s ability to recognize and respond to pathogens and malignant transformations (14, 15).

1.1.2 MHC Class I Molecules

MHC class I molecules are constitutively expressed on the surface of nearly all nucleated cells (16) and are most stable as a trimolecular complex consisting of a heavy chain (mol.wt. approx. 45-47 kD), a non-covalently associated beta-2 microglobulin (β\(_2\)m) light chain (mol.wt. 12 kD), and a peptide 8-12 amino acids in length. The heavy chain has three extracellular domains, α1, α2, and α3, each composed of approximately 90 amino
acids, a transmembrane segment of about 25 amino acids, and a cytoplasmic domain of approximately 30-40 residues (Figure 1-2). The intron/exon structure of Class I heavy chain genes closely parallels that of the of the protein domain structure (refer to Figure 2-1). The crystal structure of the class I extracellular region, resolved by x-ray crystallography (Figure 1-2), has revealed much about the overall secondary structure and the nature of its association with other polypeptide chains.

1.1.2.1 Peptide-binding Region

The α1 and α2 regions are located at the amino terminus of the class I heavy chain and are chiefly responsible for binding peptide antigens for presentation to circulating immune cells. Together they form a superdomain, which consists of an eight-stranded β-pleated sheet supporting two parallel strands of α-helix (see Fig. 1-2). The two α-helices form the sides of a cleft whose floor is formed by the strands of the β-pleated sheet (17). This cleft is of the appropriate size to contain one peptide of 8-12 amino acids in length. Sequence analysis of class I alleles has demonstrated that most of the polymorphic regions of class I genes are contained within the α1 and α2 domains and tend to be clustered in areas that form the peptide-binding cleft (18, 19). This has led to the general conclusion that polymorphism among MHC class I alleles serves to create variation in the binding properties of the peptide cleft. It has been proposed that the high level of polymorphism of MHC molecules has evolved and is maintained in each species so that members of the population express different alleles capable of binding many distinct foreign peptide antigens. This ensures that species members have a wide variety of
Figure 1-2: MHC Class I molecular structure.

(A) Side view and (B) top view of a human class I MHC molecule. The arrows represent polypeptide folded as β-pleated sheet and the coils represent polypeptide folded as α-helix. Panel B shows a close-up view of the peptide-binding cleft, formed by two parallel α-helices from the α1 and α2 domains. (C) Schematic representation of a class I molecule emphasizing its domain structure. Figure taken from “Cellular and Molecular Immunology, 3rd ed.” by Abbas, Lichtman, and Pober 1997.
peptide presentation capacities, thereby reducing the chances of a population extinction threat due to the emergence of a new pathogen.

The α1 and α2 domains of the class I heavy chain are also known to be recognized and bound by T cell antigen receptors (TcR) from CD8+ T cells and thus form the basis of CTL recognition of MHC class I molecules (18). Specific TcRs interact with both MHC-associated foreign antigens and with the MHC molecules themselves. T cell recognition and activation will be discussed in greater detail in later sections.

1.1.2.2 Immunoglobulin-like Region

The α3 domain of the heavy chain is highly conserved among all class I molecules and shows homology to Ig constant domains. This relatively nonpolymorphic region contains the binding sites for CD8 molecules and is located proximal to the plasma membrane (20).

β2m is encoded by a gene outside the MHC and is invariant in all human class I molecules examined (there are two common alleles in mice). Like the α3 segment, β2m is approximately 90 amino acids in length, contains a disulphide-linked loop, and is structurally homologous to an Ig constant domain. The HLA-A2 crystal structure shows that both the α3 domain and β2m are folded to form Ig-like domains and thus MHC class I molecules are considered to be members of the Ig gene superfamily (17, 21). The interactions of β2m with the α1, α2, and α3 domains of the heavy chain appear to be
critical for maintaining class I molecules in their native conformation. Displacement of \( \beta_2 \text{m} \) from the class I complex results in the loss of recognition by most anti-class I antisera and in lack of recognition by specific TcR. Likewise, specific peptide binding has been shown to affect class I complex formation and conformational stability (3).

1.1.2.3 Carboxyl Terminal Region

The heavy chain extends from the \( \alpha_3 \) segment into a short connecting region and then into a stretch of approximately 25 hydrophobic amino acids. This is believed to comprise the transmembrane region of the molecule, forming an \( \alpha \)-helix which passes through the hydrophobic region of the plasma membrane lipid bilayer and anchors the MHC molecule in the membrane. As with many known transmembrane proteins, the hydrophobic sequence is abruptly terminated at its carboxy terminal by a cluster of basic amino acids that are believed to interact with the phospholipid regions of the inner side of the plasma membrane. A papain cleavage site is located just outside of the transmembrane region and has been used to cleave the transmembrane and cytoplasmic domains from the extracellular portions of the molecule. While papain treatment does affect the solubility of class I molecules, it appears not to affect the overall structure of the extracellular domain.

The cytoplasmic domain of MHC class I molecules is the central topic of this thesis and will be discussed in detail in Section 1.4.
1.1.3 MHC Class II Molecules

MHC class II molecules, despite their recognition by different classes of T lymphocytes and distinct antigen presentation functions, share many fundamental similarities with class I molecules. Like class I, their extracellular region consists of four domains of approximately 90 amino acids each, two of which form a highly polymorphic peptide binding region and two of which form Ig-like domains (22). However, in contrast to class I molecules, class II molecules are composed of two non-covalently associated polypeptide chains which are similar to each other in overall structure. The α chain (32-34 kD) and β chain (29-32 kD) are encoded by separate MHC genes and each contains two extracellular domains (one for peptide binding and one Ig-like), a transmembrane segment of approximately 25 amino acids, and an intracytoplasmic region of variable length (see Figure 1-3).

Similar to class I molecules, the α1 and β1 domains interact to form a peptide-binding superdomain composed of two α-helices and eight β-pleated sheets (22). The class II peptide binding cleft is somewhat longer and is open at both ends, thereby allowing peptides of larger size (10-34 amino acids in length) to bind. The polymorphic residues tend to be concentrated in these regions, again leading to a wider diversity of peptide-binding capabilities within a population. This peptide-binding region is recognized by TcR expressed on CD4+ T lymphocytes, forming the basis for recognition of MHC class II molecules by helper T lymphocytes.
Figure 1-3: MHC Class II molecular structure.

(A) Side view and (B) top view of a human class II MHC molecule. The arrows represent polypeptide folded as β-pleated sheet and the coils represent polypeptide folded as α-helix. Panel B shows a close-up view of the peptide-binding cleft, formed by two parallel α-helices from the α2 and β1 domains. (C) Schematic representation of a class II molecule emphasizing its domain structure. Figure taken from “Cellular and Molecular Immunology, 3rd ed.” by Abbas, Lichtman, and Pober 1997.
The MHC class II α2 and β2 domains interact in a manner that is structurally homologous to that of the class I α3 domain and β2m. These nonpolymorphic regions are folded into Ig-like domains and have been shown to provide binding sites for CD4 (23). The transmembrane domains of the α and β chains are both very similar to that of the class I heavy chain, and both also contain papain cleavage sites in their extracellular regions proximal to the plasma membrane. The intracytoplasmic regions are shorter than that of class I (10-12 a.a.) but contain endocytic motifs that are important for class II antigen presentation function. This will be discussed in more detail in Section 1.3.2.

One of the most significant differences between MHC class I and class II molecules is their patterns of expression among different cell types. Whereas class I molecules are constitutively expressed on virtually all nucleated cells, class II molecules are normally expressed only on activated B lymphocytes, activated macrophages, dendritic cells (DCs), and a few other cell types (24). This distribution reflects their distinct roles in antigen presentation, which shall be outlined in more detail in the following sections.

1.2 Antigen presentation

Research on the mechanisms of antigen presentation by MHC molecules quickly revealed important distinctions between class I- and class II-restricted presentation pathways. It was found that chloroquine, an inhibitor of lysosomal proteases, could interfere with class II presentation but did not affect presentation by Class I. Conversely, protein synthesis inhibitors could interfere with class I presentation while not immediately impacting on
presentation by class II. This led to the general paradigm that MHC class I molecules present peptides derived from proteins synthesized in the cytoplasm of the cell while MHC class II molecules present peptides derived from proteins acquired from exogenous sources. This model has certain important exceptions but it is still a constructive generalization. The following sections will outline the classical MHC class I and class II antigen presentation pathways and will also describe the specialized phenomenon of exogenous presentation by class I molecules.

1.2.1 MHC Class I-restricted endogenous antigen presentation

During their biosynthesis, MHC class I molecules acquire and bind to antigenic peptide fragments derived from proteins that are made intracellularly. The MHC complex is subsequently transported to the cell surface where peptides are displayed. This is thought to be the primary means by which intracellular events such as viral infection or malignant transformation are detected by the immune system. Circulating cytolytic T lymphocytes surveying the body can detect and specifically kill cells that are displaying viral or oncogenic antigens on their surface, by recognizing them as foreign (25, 26). Since MHC class I proteins are so widely expressed, nearly all cells can be monitored and potentially recognized by these CD8\(^+\) T cells. Interferon-type cytokines, released in response to viral infections, are known to cause the transcriptional upregulation of a number of MHC genes, leading to increased class I and class II surface expression in a wide variety of cell types (3). This presumably increases the antigen presentation capabilities of the infected host cells and allows for the most efficient recognition by circulating CTLs.
The pathway that results in MHC class I-peptide presentation begins with the cytoplasmic synthesis of a foreign or self protein (see Figure 1-4). Such proteins are continuously sampled in the cytoplasm by proteasomes, multisubunit enzyme complexes that degrade proteins into short peptide fragments (27). Proteasomes are involved in both the ubiquitin-dependent, and ubiquitin-independent pathways of protein degradation (28). The proteosome complex resembles a barrel with two outer rings of seven α subunits each and two inner rings of seven β subunits each (29). The molecular spacing of the catalytic β subunits is such that the fragments produced during protein degradation are typically 8-10 amino acids in length, and it is interesting to note that class I molecules preferentially bind to peptides of this length. Intriguingly, two of the inducible low molecular weight proteins (LMP) of the proteosome, LMP-2 and LMP-7, are encoded by genes that are also part of the MHC (27, 30).

The peptides generated by the proteosome are transported into the endoplasmic reticulum (ER) by TAP-1 and TAP-2 (Transporters associated with Antigen Presentation) (24). These proteins are members of the adenosine triphosphate- (ATP-) binding cassette family of transporter proteins and are also encoded by genes in the MHC. The peptide transport complex is commonly proposed to consist of a heterodimer of TAP-1 and TAP-2, but evidence has suggested that some functional peptide transport can occur in the presence of only one of the TAP molecules (31). Both TAP-1 and TAP-2 have been shown to physically associate with MHC class I heavy chains in the ER, and these components are thought to form part of a larger multisubunit peptide-loading complex (32, 33).
Figure 1-4: MHC Class I antigen presentation pathway.

Schematic diagram depicting the MHC Class I pathway of endogenous antigen presentation. Figure taken from "Cellular and Molecular Immunology, 3rd ed." by Abbas, Lichtman, and Pober 1997.
The assembly of MHC Class I-peptide complexes in the ER is a multi-step process involving several proteins. Nascent heavy chains are cotranslationally translocated into the ER membrane by way of amino-terminal signal peptide (34). There they are immediately N-glycosylated (at one site for human class I, two sites for murine class I) and then are rapidly associated with calnexin, an ER chaperone protein (35, 36). Calnexin is thought to retain incompletely assembled heavy chains in the ER, where they subsequently associate with \( \beta_2m \) before the HC-\( \beta_2m \) complex binds to calreticulin (37, 38). Calreticulin-associated MHC Class I molecules can then associate with the TAP complex via a protein called tapasin (39).

The generation of a final stable MHC complex is dependent upon the presence of MHC Class I heavy chain, \( \beta_2m \), and peptide (40). It is only upon oligomerization of all three of these components that conformational changes occur that allow the egress of the complete trimolecular complex from the ER (41-43). The MHC complex is transported through the Golgi apparatus, where HC glycosylation groups undergo modifications, and then to the cell surface via the secretory pathway. The dependence of this process on \( \beta_2m \) and peptide is demonstrated by cell lines and knockout mice that lack TAP-1 or \( \beta_2m \) and which consequently express significantly reduced levels of class I on the cell surface (44, 45).

The elaborate control of class I surface expression is likely a reflection of the potentially serious consequences of aberrant expression, recognition, and cell killing by CTLs. It is
clearly essential to balance the ability to present viral or tumorigenic peptides with the ability to ensure that healthy cells are not indiscriminately targeted by CTLs.

It should be emphasized once more that different class I alleles have different peptide-binding affinities due to the polymorphic nature of their peptide-binding clefts. In general, peptides have two or three anchor residues that bind most strongly to the MHC Class I binding groove (46). The anchor residues are similar for all peptides binding to the same MHC molecule, but differ for peptides binding to different MHC molecules (47). The particular set of anchor residues that allow binding to a given MHC class I allele is referred to as a peptide-binding motif. Research into the nature of class I viral antigen presentation led to the observation that H-2 K\(^b\) presents one particular epitope in response to vesicular stomatitis virus (VSV) infection in a strongly immunodominant fashion (48). This epitope was later shown to be an octomeric peptide derived from the VSV nucleocapsid (N) protein, residues 52-59 (R\(^1\)G\(^2\)Y\(^3\)V\(^4\)Y\(^5\)Q\(^6\)G\(^7\)L\(^8\)) (49, 50), and helped to define the K\(^b\)-restricted motif. In the case of H-2 K\(^b\), the specific binding motif requires a tyrosine or phenylalanine at peptide residue 5 or 6 in addition to a leucine at residue 8 or 9. Despite this requirement, there remain thousands of different peptides that can potentially bind to K\(^b\) and ultimately be presented to specific CTLs.

1.2.2 MHC Class I and CD8\(^+\) T cell lymphocytes

As T lymphocytes are the effector cells that ultimately respond to antigen presentation by class I molecules, it is appropriate at this point that an overview of T cell development
and activation is presented. Since the focus of this thesis is MHC class I, the discussion will emphasize CD8\(^+\) T cell selection and antigen recognition.

T and B lymphocytes share a unique propensity for specialized genetic rearrangements that ultimately lead to a staggering array of different antigen specificities throughout the respective cell populations. In the case of B lymphocytes, it is the immunoglobulin genes that undergo rearrangements, such that each B cell produces one type of antibody molecule with a unique antigen-binding specificity (reviewed in (51, 52)). In T cells, it is the T cell receptor (TcR) genes that undergo similar rearrangements, such that each individual T cell expresses TcR with unique antigen recognition specificities (53). Recombinase enzymes encoded by the RAG-1 and RAG-2 genes mediate both types of rearrangements (54, 55). This genetic shuffling results in tens of millions of different antigen specificities for each receptor type. This broad diversity is believed to play an important part in the ability of the immune system to withstand a wide variety of pathogenic challenges. However, because one of the immune system's prime directives is to recognize the distinction between self and non-self (or altered-self), it must exert a strict level of control over which lymphocytes will ultimately form the repertoire of immune cells (56). Thus, mechanisms of deleting cells expressing self-reactive or non-functional antigen receptors have evolved to achieve this end. In the case of T cells, MHC molecules play fundamental roles at several stages of development, including repertoire selection, antigen recognition, activation, clonal expansion, and formation of immunological memory (Figure 1-5).
1.2.2.1 CD8⁺ T cell selection

The repertoire of mature T cells is shaped by positive and negative selection events that take place in the thymus following the migration of immature T cells from the bone marrow. The “thymic education” process of positive selection ensures that the T cell repertoire becomes self MHC-restricted, that is, their TcRs can recognize foreign antigens only in the context of self MHC molecules (57). A parallel process of negative selection ensures that T cells expressing TcRs with no affinity for self-MHC or expressing potentially autoreactive TcRs are eliminated or inactivated (58).

The currently accepted T cell selection model describes a process by which the specific interactions between the TcRs of developing thymocytes and the MHC molecules expressed by the thymic epithelium ultimately determine the fate of the T cells (59, 60). Under this model, if the avidity of the TcR for self MHC is extremely low (reflecting lack of self MHC recognition) or is very high (suggesting recognition of self antigens in the context of self MHC), then the T cell expressing that TcR will be deleted or inactivated. If the TcR demonstrates a low to moderate avidity for self MHC (but which could potentially undergo high-avidity interactions with complexes of foreign peptides and self MHC), a positive selection signal will be delivered to the developing T cell resulting in its maturation and emigration from the thymus to peripheral lymph nodes (61, 62). The exact nature of these positive and negative signals is still a matter of speculation, but thymic hormones produced by thymic epithelial cells have been postulated to promote T cell maturation by delivering signals to developing thymocytes that can either promote growth and survival or trigger apoptosis and cell death (63, 64).
Although immature thymocytes nearly all pass through a stage where both the CD4 and CD8 co-receptors are expressed (CD4⁺CD8⁺), an important consequence of thymic education is that the expression of one molecule is shut down such that nearly all mature peripheral T cells are either CD4⁺CD8⁻ or CD4⁻CD8⁺ (65, 66). Positive thymic selection results in CD4⁺ T cells becoming exclusively class II-restricted while CD8⁺ T cells become class I-restricted. It has been proposed that these restrictions arise as a result of the ability of CD4 and CD8 molecules to bind to class II and class I molecules, respectively, thereby promoting stronger interactions between thymocytes and thymic epithelial cells expressing these MHC molecules (67, 68).
Figure 1-5: MHC Class I involvement in T cell development.

Schematic diagram illustrating the influence of MHC Class I molecules on CD8+ T cell development at multiple levels. Class I molecules play key roles in CD8+ T cell selection, activation, proliferation, and killing responses in vivo.
1.2.2.2 CD8⁺ T cell recognition and activation

Every naïve peripheral CD8⁺ T cell has the potential to undergo activation and clonal expansion in response to TcR recognition of foreign antigens bound to class I molecules. However, this activation and proliferation can occur only if the antigen is initially presented to the T cells by a special class of cells referred to as professional antigen presenting cells (APCs). This grouping includes B lymphocytes, macrophages, and dendritic cells. All of these cells share the ability to acquire and process antigens that originate in the extracellular milieu. These cells also express co-stimulatory molecules which, combined with MHC class I-peptide recognition by TcRs, deliver signals which are considered essential for initiating CD8⁺ T cell activation. If a T cell is stimulated through binding of its TcR to an MHC-peptide complex in the absence of co-stimulatory signaling, it undergoes functional inactivation, known as anergy (69). Professional APCs are therefore considered central to the induction of T cell-mediated specific immunity, in that they provide the primary stimulus for all T cell responses. Dendritic cells (DCs) have emerged as the most potent of the APCs, and are now known to be considerably more efficient at stimulating primary T cell responses than B cells or macrophages (70, 71). DCs will be discussed further in Section 1.2.4.

The TcR is part of a multi-protein complex that includes CD3 chains. The binding of the TcR complex to an antigen-MHC complex on APCs generates intracellular signals that, combined with costimulatory signals through CD28 (generated by binding either of its receptors CD80 or CD86), transiently increase the transcription of several genes that are normally quiescent (72) (73). The T cells begin to express higher levels of the IL-2
receptor in addition to the T cell growth factor IL-2. These autocrine signals, in addition to signals from several CD4+ T cell-derived cytokines, cause T cells to enter a rapid proliferation phase known as clonal expansion (74, 75). Most of the progeny of antigen-responsive cells go on to perform effector functions such as CTL killing, but some develop into antigen-specific memory T cells. These memory cells are very long-lived and are capable of initiating larger and more rapid secondary immune responses upon subsequent exposure to antigen (76).

Mature CTLs, once activated and expanded by professional APCs, require only recognition of the MHC-antigen complex to trigger CTL killing (77). CTL-mediated killing involves two complementary mechanisms: first, granule exocytosis of a membrane pore-forming protein (perforin) that causes osmotic lysis of target cells; secondly, secretion of cell toxins (lymphotoxin, granzyme B, or related molecules) that activate DNA-degrading enzymes and trigger apoptosis of target cells. Perforin possibly facilitates the release of these substances from vesicles after they have been endocytosed by target cells. Despite the requirement for cell contact between CTLs and cells bearing MHC-antigen complexes, CTLs themselves are not injured during target cell lysis and each individual CTL is capable of killing multiple target cells (78). Due to the nearly ubiquitous nature of Class I, the clonally expanded CD8+ CTLs are able to circulate through and survey the entire body for any infected or diseased cells to prevent further pathogenesis. This immunosurveillance and killing continues until the infection or disease process is brought under control at which time most of the CTL, presumably due to lack of stimulation by antigen, undergo apoptosis (79).
1.2.3 MHC Class II-restricted exogenous antigen presentation

Though not a central topic of this thesis, it is nonetheless useful to briefly outline class II-restricted antigen presentation of exogenous antigens. Early in the biosynthesis of class II molecules, the \( \alpha \) and \( \beta \) chains bind to the invariant chain (Ii) to form a nonameric protein complex consisting of three copies of each polypeptide chain (80). This complex is unable to bind to peptides in the ER due to the Ii physically blocking the peptide-binding groove formed by the \( \alpha \) and \( \beta \) chains (81). Intracellular targeting sequences in the Ii chain direct the complex to the cell surface by a route that intersects with the endocytic pathway (see Figure 1-6) (82, 83). It is believed that specialized endocytic compartments termed MIICs are where class II antigenic peptide loading takes place (84, 85). Within these compartments, Ii is cleaved from the class II complex, initially leaving behind an Ii fragment (termed CLIP) in the peptide-binding cleft. CLIP is subsequently removed by the DM protein (a class II-related protein resident in MIICs), thereby allowing empty class II molecules to bind to the peptides present in the endocytic compartment (86). Peptide-bound class II molecules are then transported to the cell surface where they present antigens to CD4\(^+\) T cells. Since most of the peptides within the endocytic pathway tend to be derived from extracellular proteins that have undergone lysosomal degradation, the majority of peptides displayed by class II are derived from exogenous sources (87).

Whereas Class I-restricted antigen presentation generally alerts the immune system to the presence of intracellular pathogens and altered self-components, class II-restricted presentation is historically thought to be more important for alerting the immune system
to the presence of extracellular pathogens (ie. bacteria and parasites) as well as foreign protein antigens. These predispositions are reflected in the peptide pools to which the MHC molecules are able to gain access (88). Antigen presentation by Class II molecules is essential for activating CD4\(^+\) helper T cells, which are instrumental in the development and amplification of a wide variety of immune responses. Activated CD4\(^+\) cells play a key role in influencing these responses largely through the release of cytokines that can activate macrophages, influence B cell activation and antibody production, and enhance CTL development, along with many other functions (89, 90).
Figure 1-6: MHC Class II antigen presentation pathway.

Schematic diagram depicting the MHC Class II pathway of antigen presentation. CLIP, Class II-associated invariant chain peptide. Figure taken from “Cellular and Molecular Immunology, 3rd ed.” by Abbas, Lichtman, and Pober 1997.
1.2.4 Presentation of exogenous antigens by MHC Class I molecules

The general paradigm that has emerged for class I molecules is that they present antigens derived strictly from endogenous sources. This has been confirmed experimentally in a wide variety of cell types and furthermore makes sense intuitively. Obviously, since class I-antigen complexes form targets for killing by CTL, it would be counterproductive and more likely dangerous to allow class I molecules to indiscriminately bind and display peptides from exogenous sources. However, as mentioned above, T cell activation requires that antigen presentation be performed exclusively by professional APCs.

Dendritic cells (DCs) are the most potent APC known, and can stimulate vigorous activation of both CD4\(^+\) and CD8\(^+\) T cells \textit{in vitro} at very low cell concentrations (91). They can express very high levels of both Class I and Class II molecules and are considered to be central to the initiation of immune responses \textit{in vivo}, despite their sparse numbers (comprising 0.5 to 1% of total leukocytes). In the body, DCs form an interactive network of cells that can migrate between lymphoid organs (spleen, lymph nodes, Peyer’s patches, etc.), lymphatics, blood, skin (as Langerhans Cells (LCs)), and various other organs (heart, liver, and lung). The presence of DCs at all of these sites functions as an effective antigen sampling system to monitor the entire body for foreign molecules, infection, or tumorogenesis (92-94).
DCs arise from bone marrow-derived myeloid precursor cells that can also give rise to granulocytes and macrophages (95). Extensive dendritic processes, amorphous nucleus, motile nature, and a lack of classical endocytosis separate DCs from macrophages (96). Their extensive antigen sampling is accomplished by fluid phase uptake that proceeds via macropinocytosis. The uptake of large vesicles permits the uptake of up to one cell volume of fluid per hour per DC (97). The macropinocytotic vesicles are transported into the cell where some of their contents fuse with MIIC compartments (98).

Generally, immature DCs in the skin and tissues are extremely efficient at continuously sampling the extracellular fluid for foreign antigen. Upon acquiring antigen, DCs begin to mature and migrate to nearby lymphoid organs. During this maturation process, DC physiology changes from one of antigen uptake and processing to enhanced T cell stimulatory capacity (99, 100). Reflecting these changes, DCs begin to upregulate the surface expression of several molecules involved in antigen presentation. These include MHC class I and II molecules, co-stimulatory molecules CD80 and CD86 (also known as B7-1 and B7-2, both ligands for CD28), and other molecules involved in cell adhesion and modulation of DC function (101-105). High levels of Fcγ receptors ensure that immune complexes are preferentially internalized, processed and efficiently presented to both CD4+ and CD8+ T cells (106). DCs have the capacity to efficiently present processed antigen on both class I and class II molecules and can also maintain intracellular stores of antigen for extended periods of time (107).
Presentation of exogenously-derived antigens by class I molecules, while going against the general paradigm, seems to play an fundamental role in the initiation of CD8\(^+\) T cell responses by APCs. It has been known for some time that CTL responses can be generated \textit{in vivo} by immunization with protein antigens, inactivated viruses, allogeneic transplanted cells and tumours (108-111). CD8\(^+\) T cell responses are also observed for bacteria and parasites that do not enter the cytoplasm. Under the general paradigm it is difficult to explain how these kinds of ‘cross-priming’ immune responses are possible, especially considering that these antigens cannot lead to intracellular production of foreign proteins. Even in the case of active viral infections, under the paradigm APCs themselves would have to be infected in order to achieve effective class I antigen presentation. While possible, it is unlikely to be the most efficient or effective method of initiating CTL responses \textit{in vivo}.

While the exact mechanisms of exogenous presentation by MHC class I remain to be elucidated, an endosome-to-cytosol pathway has been proposed to exist in dendritic cells and possibly macrophages (112-115). Under this model, antigens acquired exogenously are transported into the cytoplasm, where they undergo classical proteosome degradation and TAP-dependent peptide loading onto class I molecules (116, 117). Other examples of exogenous antigen presentation have been shown to occur in a TAP-independent manner, suggesting that alternate pathways may exist (118-121). The unpublished observation that high levels of class I molecules are found within MIIC-like vesicles in DCs (personal communication, R. Steinman) suggest that class I molecules may access the endocytic pathway and therefore may acquire antigenic peptides at sites other than the ER.
Class I molecules have been reported to undergo rapid internalization via coated pits in macrophages and monocytes. In these cells, coated vesicles containing class I molecules are transported through endosomes to the trans-Golgi where they are presumably recycled back to the cell surface (122). Since macrophages are also known to present exogenous antigens in the context of class I molecules (123), the paradigm of strictly endogenous antigen presentation by class I will likely have to be modified to account for the behavior of professional APCs.

1.3 Cytoplasmic phosphorylation, trafficking & endocytic motifs

Because the focus of this thesis is the cytoplasmic tail of Class I molecules, the discussion at this point will switch from antigen presentation to a brief general overview of what is known about the cytoplasmic domains of transmembrane proteins. Emphasis will be placed on endosomal trafficking and internalization motifs and will include the role of phosphorylation in these processes.

1.3.1 Phosphorylation

Protein phosphorylation is one of the best-studied post-translational modifications. Phosphorylation of tyrosine, serine, and threonine residues has been shown to be involved in numerous cell processes including transmembrane signaling, cytoplasmic signal transduction, transcription factor activity, cell cycle control, and intracellular
trafficking. Protein phosphorylation can mediate conformational changes, enzyme activity, sub-cellular localization, and protein-protein interactions. As such, a complete review of these processes is beyond the scope of this thesis. Instead, a few pertinent examples should serve to convey the general functional implications, mechanisms, and control of protein phosphorylation.

Protein phosphorylation is regulated by enzymes known as kinases and phosphatases. Kinases (which add phosphate groups to target proteins) are generally divided into two categories, tyrosine (Tyr) kinases and serine/threonine (Ser/Thr) kinases. Tyr kinases are often split into receptor-like transmembrane or cytoplasmic sub-categories. The EGF receptor was one of the first to be characterized and may be considered a prototype receptor Tyr kinase. This receptor, upon binding its ligand EGF, undergoes dimerization and autophosphorylation (124, 125). This in turn initiates downstream signaling by recruiting and activating other signal transduction components, including Ser/Thr kinases (126-128). Receptor-mediated phosphorylation is often the first step in a cascade involving multiple proteins that phosphorylate each other in sequence and result in transduction of a signal that can ultimately drive intracellular Ca$^{2+}$ fluxes, gene transcription, cellular activation, and cell division (129-132).

Cytoplasmic Tyr kinases, despite their localization, are often linked to plasma membrane signal transduction (133). Among other functions, these kinases are known to phosphorylate the Tyr residues on Immunoreceptor family Tyrosine-based Activation Motifs (ITAMs) which have the sequence $[D/E-X_7-D/E-X_2-Y-X_2-L/I-X_7-Y-X_2-L]$
In TcR-mediated T cell activation, the Tyr kinase p56	extsuperscript{ck} likely phosphorylates ITAMs present on the CD3 ε, γ, δ, and ζ chains. These phosphorylated ITAMs are then able to recruit SH2 domain-containing proteins, including phosphatidyl inositol 3-kinase and ZAP-70, to the TcR complex (135). This results in the generation of inositol-1,4,5-triphosphate (IP₃) and diacylglycerol, which in turn lead to intracellular Ca²⁺ flux, and protein kinase C (PKC) activation, respectively. These events ultimately lead to IL-2 gene transcription and T cell activation (74, 136).

Major Ser/Thr kinases include PKC, PKA, Raf kinases, and MAP kinases. The MAP and Raf kinases are involved in downstream signaling from plasma membrane events and are integral components of the phosphorylation cascade-signaling network of proteins alluded to above (137). Many different types of membrane signals can activate this group of proteins, including receptor-ligand binding and TcR engagement (74, 138). Raf-1 was originally characterized as a proto-oncogene (139). Upon activation, Raf-1 is able to phosphorylate and thereby activate the MEK kinase, which in turn phosphorylate the ERK family of MAP kinases resulting in their activation (127, 128, 140). The ERK kinases phosphorylate and activate Ets domain-containing transcription factors such as Elk-1.

PKC has multiple isoforms and most can be activated by the production of diacylglycerol (143). Activated and membrane-localized PKC can influence cellular metabolism by phosphorylating and thereby modulating the function of a variety of membrane proteins. For example, PKC-mediated phosphorylation of the Na⁺/H⁺ antiporter enhances the
activity of this ion exchange system, resulting in increased intracellular pH (144, 145).
PKC is also known to phosphorylate the EGF receptor at a cytoplasmic threonine residue
(146), resulting in the inhibition of the endogenous receptor kinase activity, reduced
affinity of the receptor for its ligand, and the promotion of receptor internalization (147-
149). Similarly, PKC phosphorylation of the insulin receptor β subunit inhibits the endogenous receptor kinase activity and reduces its ability to bind insulin (150, 151).

The intracellular localization of many different proteins has been shown to be phosphorylation-dependent. For example, the polymeric immunoglobulin receptor (plgR), which mediates the transport of IgA and IgM across a variety of epithelia, requires a serine phosphorylation event for efficient transcytosis from the basolateral to the apical cell surface (152, 153). This phosphorylation event is believed to provide a sorting signal that allows the cell to distinguish ligand-bound receptors from empty ones, consequently ensuring that only loaded receptors are transported to the apical surface for ligand release. Cytoplasmic phosphorylation of several membrane proteins is known to promote their internalization from the cell membrane. These include the transferrin receptor (154), the EGF receptor (155), the asialoglycoprotein receptor (156), the erythropoietin receptor (157), and the insulin receptor (158), to name but a few. Phosphorylation as a transport signal is a common theme in cell biology and provides a means of efficient quality control for several membrane receptors.

Phosphatases (which remove phosphate groups from target proteins) counteract and balance the activity of kinases, thus providing for the cyclical nature of cellular signal
transduction mechanisms (159, 160). They can also initiate cellular processes such as T cell activation, as has been reported for the receptor tyrosine phosphatase CD45. Dephosphorylation of the negative regulatory site (Tyr-505) of p56\textsuperscript{ck} by CD45 causes activation of p56\textsuperscript{ck} (161), leading to phosphorylation of ITAM motifs on CD3 chains and further downstream signaling events.

This brief discussion of protein phosphorylation should underscore the importance of this post-translational modification in multiple cellular processes. The coordinated regulation of such a vast and complex network of kinases and phosphatases is only now starting to be understood in detail. While the overall picture has yet to be fully delineated, useful information has been gleaned from the study of individual proteins. By studying the cytoplasmic domains of a variety of membrane proteins, common motifs have been identified which have provided valuable clues to the mechanisms underlying cell signaling (eg. ITAMs, discussed above) and intracellular trafficking. Motifs involved in the cellular transport and localization of membrane proteins will be discussed in the following section.

### 1.3.2 Cytoplasmic Trafficking Motifs

The trafficking and intracellular localization of several integral membrane proteins has been shown to depend upon the cytoplasmic regions of these proteins. Cytoplasmic domains frequently contain signals in the form of amino acid motifs that are recognized by other cellular components. These components can mediate either retention of the
protein in, or transport to certain organelles, depending on the nature of the signal. For example, two virally-encoded integral membrane glycoproteins, herpes simplex virus gB-1 and vesicular stomatitis virus G protein, have been shown to be dependent on their cytoplasmic regions for trafficking from the ER to the cell surface (162, 163). By contrast, the ER resident chaperone protein BiP contains the well-known KDEL motif as part of its cytoplasmic domain. This motif is sufficient to retain BiP in the ER and prevent its transport out to the cell surface via the secretory pathway (164, 165). Similarly, the adenovirus E3-19K protein contains signals in its cytoplasmic tail that mediate its ER localization, thereby allowing it to perform its immunosubversive function of binding and retaining class I molecules (166). These are but a few of the numerous examples of transmembrane proteins dependent upon cytoplasmic domain sorting signals for transport and intracellular localization.

Two distinct cytoplasmic sorting motifs are generally recognized as being important for directing membrane proteins from the trans-Golgi network (TGN) or the plasma membrane into the endosomal system (Figure 1-7). Di-leucine-based determinants (usually containing an LL motif) have been implicated in directing proteins such as the mannose-6-phosphate receptor (MPR), CD3 \( \gamma/\delta \) chains, limp-II, the invariant chain (Ii), and MHC class II into endosomal compartments via clathrin-coated vesicles (CCVs) (167-174). In some cases (MPR, Ii, and MHC class II), this di-leucine motif is associated with an adjacent phosphorylation site, and phosphorylation of this site is a prerequisite for delivery of the proteins into the endocytic pathway (175, 176).
Tyrosine-based motifs (YXXZ or NXXY, where Z represents a bulky hydrophobic amino acid) are also implicated in endosomal sorting and are thought to be responsible for directing several membrane proteins, including CD1b, lamp-1, lamp-2, lysosomal acid phosphatase (LAP), the transferrin receptor (TfR), the asialoglycoprotein receptor, the insulin receptor, and the epidermal growth factor receptor (EGFR) into CCVs (154-156, 177-180). In some cases (ie. EGFR, TfR), phosphorylation of the tyrosine residues is required for internalization of membrane proteins into endosomal compartments. Recycling of certain receptor proteins back to the cell surface following ligand dissociation may also be mediated by such phosphorylation events (181, 182).

Recent evidence has shown that interactions between tyrosine- and leucine-based sorting signals in the cytoplasmic tails of transmembrane proteins and adaptor complexes containing AP-1 and AP-2 are the first steps in the formation of CCVs that deliver proteins to their intracellular destination (183). Several in vitro studies have demonstrated interactions between medium (mu) chains of AP-1 and AP-2 with both types of sorting signals (173, 184). The binding of adaptor complexes to tyrosine-based or di-leucine-based signals provides a structural basis for the triskelion clathrin molecules to form the lattices that are essential for the formation of CCVs. Substitution of di-leucine sequences in the Ii chain abolishes AP-1 binding (185). Interestingly, mutation of the Ii serine residue 9, a site of phosphorylation, also abolishes AP-1 binding. Phosphorylation of this residue is required for the Ii-mediated trafficking of MHC class II to the endocytic compartment (186). Furthermore, substitution of the di-leucine motif in the MHC class II cytoplasmic tail greatly reduces antigen presentation by class II molecules (174).
Presumably, class II molecules are dependent on these di-leucine motifs for trafficking through peptide-containing compartments and subsequent antigen presentation on the cell surface.

MHC class I molecules share several features with the transmembrane proteins discussed above, including a site of phosphorylation and a sequence reminiscent of a tyrosine-based endocytic motif (187, 188). The next section will focus in detail on the cytoplasmic domain of class I molecules and will set the stage for the general rationale and approach used in this thesis.
Two major sorting signals, di-leucine-based and tyrosine-based, are known to be primarily responsible for directing integral membrane proteins from the plasma membrane or the TGN into endosomes. AP-1 and AP-2 adaptor complexes recognize and bind to these motifs as one of the initial steps in the formation of clathrin-coated vesicles (CCVs), which transport the proteins through endosomal compartments. Examples of proteins that contain endocytic motifs in their cytoplasmic domains are listed. Asterisks (*) indicate proteins for which phosphorylation of its cytoplasmic domain is a prerequisite for trafficking or internalization. The HIV-\textit{ nef} protein is able to subvert the normal trafficking of both CD4 and MHC class I molecules, acting to down-regulate their cell surface expression by increasing their accumulation into CCVs. This action of \textit{ nef} is dependent upon the di-leucine motif of CD4 and the conserved cytoplasmic tyrosine residue of MHC class I.
1.4 MHC Class I cytoplasmic domain

1.4.1 Previous findings

While much has been learned about the extracellular domain of MHC class I molecules, relatively few detailed studies have been conducted on the intracellular region. The class I cytoplasmic domain is known to be encoded by three separate exons, VI, VII, and VIII (also called (Intracytoplasmic) I₁, I₂, and I₃), and is typically 30-35 amino acids in length. Comparison of the amino acid sequences of MHC class I cytoplasmic domains of different species reveal the striking conservation of a number of amino acids (Figure 1-8). In particular, the tyrosine residue encoded in exon VI, and two or three serine residues encoded by exon VII are highly conserved in all mammals and in species as evolutionarily distant as the chicken, shark and zebrafish (189-205).

Previous studies on class I molecules showed that the cytoplasmic tail was constitutively phosphorylated in human peripheral blood leukocytes and in a lymphoblastoid cell line (206). This site was later mapped to one of the well-conserved serine residues (Ser-335), although nearby Ser-332 could not be ruled out as an additional or alternate phosphorylation site (187). Reportedly, only a small proportion of class I molecules is phosphorylated in normal cells (207), but phosphorylation can be upregulated by treatment of cells with phorbol esters (208). Class I molecules were subsequently shown to be phosphorylated in vitro by cyclic AMP-dependent protein kinase (209) and by protein
<table>
<thead>
<tr>
<th>Species</th>
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<th>Reference</th>
</tr>
</thead>
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<td>Mouse</td>
<td>-RRRNTGGKGGGDYA_ALAPG_SQTSDLSLPDCKV</td>
<td>(189)</td>
</tr>
<tr>
<td>Rat</td>
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<td>(192)</td>
</tr>
<tr>
<td>Cat</td>
<td>-RKKFSGKGP_YTRAARRD_STQGSDSVMAPKV</td>
<td>(193)</td>
</tr>
<tr>
<td>Horse</td>
<td>-RKKRSGEKRG_YVQAANS_DAQGS_SVPQ_KV</td>
<td>(194)</td>
</tr>
<tr>
<td>Sheep</td>
<td>-RKKCSSDGK_IQASS_D_SAQGS_DVSLTVKPV</td>
<td>(195)</td>
</tr>
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<td>Bovine</td>
<td>--KKKSGGKGGY_TQASS_D_SAQGS_DVSLTACKV</td>
<td>(196)</td>
</tr>
<tr>
<td>Gorilla</td>
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<td>Z-fish</td>
<td>--KKKSGGKGG_IQASS_D_SAQGS_DVSLTACKV</td>
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</table>

Figure 1-8: MHC Class I cytoplasmic tail species homology alignment.

Aligned amino acid sequences of MHC Class I cytoplasmic domains from a variety of vertebrate species. Highly conserved tyrosines and serines are highlighted in bold print. The species and MHC Class I alleles are listed as they appear from top to bottom: mouse *Mus musculus* (H-2Kb), brown rat *Rattus norvegicus* (RT1-AA), rabbit *Oryctolagus cuniculus* (RLA 11/11), dog *Canis familiaris* (DLA A9/A9), domestic cat *Felis catus* (FLA-2), horse *Equus caballus* (EX-MHC-B1), sheep *Ovis aries* (SHP-MHCE), cow *Bos taurus* (BOLA BL3-6), gorilla *Gorilla gorilla* (GOGO-A0101), human *Homo sapiens* (HLA-A0101), red-necked wallaby *Macropus rufogriseus* (MARU UB-01), harbour seal *Phoca vitulina* (PLA-A1), chicken *Gallus gallus* (B-FIV), African clawed frog *Xenopus laevis* (Xen-MHC I), reptile *Ameiva ameiva* (MHC I), leopard shark *Triakis scyllium* (UAA), and zebrafish *Brachydanio rerio* (UBA-01).
kinase C (210), though neither event has been confirmed to occur naturally.

Intriguingly, alternative mRNA processing in many cell types can splice out the exon (exon VII) encoding for this phosphorylation site, and this has given rise to speculation that splicing may result in functional modification of class I molecules (211-213). Evidence for this was provided when it was demonstrated that human class I molecules containing deletions of exon VII were not internalized to the same extent as wild-type molecules in a T lymphoblastoid cell line (188).

MHC class I molecules containing truncations of the entire cytoplasmic domain are well-expressed at the cell surface in all transfected cell types examined (208, 214) and show similar maturation rates. Furthermore, their abilities to function as in vitro targets for allogeneic or specific CTL remain generally unaffected by cytoplasmic tail truncations (214), although there is some evidence to the contrary (215). It is known that class I molecules undergo rapid constitutive internalization into clathrin-coated pits in activated T cells (216, 217) and in macrophage/monocyte cell types (122). Attempts to correlate class I phosphorylation with its internalization have shown that while phorbol ester-induced phosphorylation could occur in all cell types examined, only lymphoid cells were seen to subsequently internalize class I molecules at significant rates. Class I molecules containing deletions or gross substitutions of the cytoplasmic tail could not be internalized in this fashion (208).
The conserved cytoplasmic tyrosine residue encoded by exon VI was first noted for its in vitro phosphorylation by the Src tyrosine kinase (218), although tyrosine phosphorylation has never been reported for class I molecules isolated from cell lines or animal tissues. Additionally, deletions of exon VI were shown to have a negligible effect on class I internalization in a T cell line (188). However, the tyrosine residue was shown to be inaccessible to radioiodination during transport of MHC class I to the cell surface, implying that it may play a role in intracellular trafficking (219). More recently, this tyrosine was shown to be essential for the HIV nef protein to down-regulate cell surface expression of human class I molecules in nef-transfected cells (220, 221).

1.4.2 Proposed functions

Many possible functions have been postulated for phosphorylation of the cytoplasmic domain of MHC class I, including cytoskeletal anchoring, oligomerization, β2m binding, intracellular trafficking, and co-stimulatory signaling mechanisms (151, 219, 222-225). Despite this speculation, very few solid answers have emerged regarding the true function of the cytoplasmic tail that would explain its high degree of conservation. Intracellular signaling through cross-linking of surface class I molecules has been shown to occur in T cells (226-228). However, the resulting phosphorylation cascade and Ca\(^{2+}\) flux could also be demonstrated in T cell lines expressing class I molecules with entire cytoplasmic truncations (229). Signaling was later shown to be mediated by the CD3 chain in response to class I cross-linking (230). The significance of this T cell-specific
phenomenon remains debatable and, more importantly, it has shed no further light on the function of the class I cytoplasmic tail.

Another T cell-specific phenomenon, which does provide a clue as to the true function of the cytoplasmic domain, is the rapid constitutive internalization and recycling through endosomal compartments of class I molecules (216, 217). Up to 50% of surface class I has been shown to internalize per hour in activated T cells (231). From previous studies, it is clear that determinants in exon VII are important for this process (188). What is unclear, however, is the significance of the process itself. In particular, it is difficult to explain why T cells would expend considerable energy internalizing and recycling their own class I molecules when their stimulation patterns and effector functions seem to be governed by class I molecules that are expressed on other cells.

One suggestion put forward is that the low pH (5.0-5.5) of endosomal compartments is appropriate to facilitate peptide exchange. This hypothesis, based on the experimental observation that \(\beta_2\)m and peptide can dissociate from the class I heavy chain at lower pH (232), has been proposed to explain the recycling function of class I molecules (233). Although there has been some evidence to support this idea (234), very little is currently known about the ultimate consequences of class I antigen presentation by T cells, especially in vivo. This makes it difficult to speculate on what the physiological and immunological significance of T cell class I recycling might be.
While this process remains somewhat of an enigma in T cells, it can occur in other cell types as well. Macrophages are also known to internalize MHC class I molecules via coated pits and possibly recycle them back to the cell surface (122). These cells can also present exogenous antigens in the context of class I molecules (123). It is possible that the class I cytoplasmic tail will provide one of the critical connections between these two processes.

Ultimately, the piece of the class I puzzle that appears to be missing is the connection between the cytoplasmic domain and the antigen presentation function mediated by the extracellular region. Previous studies have, in large part, shown that the cytoplasmic domain is dispensable for antigen presentation. However, the majority of these studies have based their conclusions solely on class I antigen presentation in transfected cell lines. A more recent study characterized the CTL responses of transgenic mice expressing a class I D\textsuperscript{b} transgene that had its transmembrane and cytoplasmic exons replaced by a glycosylphosphatidylinositol (GPI) anchor-encoding sequence (235). These mice were unable to generate CTL responses to a D\textsuperscript{b}-restricted immunodominant epitope of influenza A virus. This data suggests that the cytoplasmic domain and/or the transmembrane region is essential for the \textit{in vivo} generation of at least some class I-restricted CD8\textsuperscript{+} T cell responses.
1.5 Project rationale and general approach

The focus of this research was to add to our understanding of the function of the MHC class I cytoplasmic domain. At the start of this work, several questions regarding the function of the cytoplasmic region remained unanswered. For example, what is the functional significance of its serine phosphorylation? Does phosphorylation play a role in the intracellular trafficking of class I molecules, its ability to bind to β2m, its maturation rate, or its internalization from the plasma membrane? What is the significance of the exon VII-deleted naturally-occurring class I splicing isoform? Does the cytoplasmic domain play any role in the antigen presentation function of class I molecules? How is antigen presentation in vivo affected by cytoplasmic tail mutations? Is the conserved tyrosine residue part of a tyrosine-based endocytic motif? And finally, what process(es) does the class I intracellular domain mediate that have led to its high level of conservation across a wide variety of species?

With these questions in mind, a site-directed mutagenesis strategy was employed to introduce specific mutations in the cytoplasmic domain of a well-studied murine class I allele, H-2 K^b. Details of the site-directed mutagenesis approach are outlined in Chapter 2.

1.5.1 Rationale

Amino acid conservation between species is often a good indication of the importance of particular residues in the function of a given protein domain. In general, the more
conservation shown, the more important the amino acids are. The high levels of conservation that are observed for certain elements of the class I cytoplasmic tail (Figure 1-8) played a large part in the rationale for this thesis work.

The conservation demonstrated by the class I cytoplasmic serine residues, in addition to their constituting a site of phosphorylation, made them particularly attractive targets for site-directed mutagenesis. Since phosphorylation has been repeatedly shown to be an important modulator of protein function, we were interested in exploring whether non-phosphorylatable class I molecules displayed altered intracellular trafficking and/or antigen presentation function. The intriguing natural occurrence of splicing isoforms lacking exon VII (and thus the conserved serine residues) suggests that functional modification may occur in such class I molecules and could reflect an underlying physiological control mechanism.

The highly conserved class I cytoplasmic tyrosine residue is reminiscent of tyrosine-based endocytic motifs commonly found in other membrane proteins and therefore was another attractive target for site-directed mutagenesis. Tyrosine-based endocytic motifs are classically defined as YXXZ, where Z is a bulky hydrophobic amino acid (236). In class I molecules, the cytoplasmic YXXA sequence is nearly invariant (Figure 1-8). The alanine at the Y+3 position is not bulky but is hydrophobic, implying that it could possibly constitute an alternative tyrosine-based endocytic motif.
Endocytic motifs have been shown to be essential for antigen presentation function in at least two other related systems. As mentioned earlier, di-leucine motifs in the cytoplasmic tails of MHC class II molecules and Ii are critical for class II antigen presentation (171, 174). Likewise, the MHC class I-like molecules CD1b and CD1d have been shown to contain cytoplasmic tyrosine motifs that are essential for their presentation of glycolipid antigens (237, 238). In all of these cases, deletion of the endocytic motifs leads to a failure of these molecules to access intracellular compartments where they normally acquire antigens. A serine phosphorylation event is also required to transport class II molecules to endocytic MIIC compartments (186).

MHC class I molecules share many features with the molecules listed above. In addition to containing a putative endocytic motif and a phosphorylation site, class I molecules are known to present exogenously-derived antigens. They are also internalized in clathrin-coated vesicles (CCVs) to early endosomes before recycling to the cell surface in some cell types. Binding of AP-1 and AP-2 adaptin proteins are known to be one of the initial events in the formation of CCVs, and their interactions with tyrosine- and di-leucine-based endocytic motifs have been well-documented (239, 240). In vitro studies on the HIV-\textit{nef} protein have provided some further insights into the potential dynamics of this system.

HIV-\textit{nef} has the ability to down-regulate surface expression of both CD4 and MHC class I molecules (241-243). It was recently shown that \textit{nef} interacts with the mu (medium) subunit of AP adaptor complexes, and that the down-regulation of MHC class I
molecules is dependent upon the tyrosine residue present in the cytoplasmic tail of Class I (220). Likewise, CD4 down-regulation by nef has been shown to be dependent on the di-leucine motif present in the cytoplasmic tail of CD4 (244). This down-regulation is mediated by increased accumulation of these membrane proteins into CCVs (245) (Figure 1-7).

The nef data provides some evidence that the class I cytoplasmic tyrosine is part of an endocytic sorting signal. It appears to have been uncovered only in the presence of HIV-nef, and has never been shown to be physiologically relevant in a normal, healthy system. However, its high level of conservation is compelling evidence that an important mechanistic function is dependent on the tyrosine residue. The striking similarities with other antigen-presenting molecules make a compelling circumstantial case for the Class I tyrosine-based endocytic motif to be linked to its antigen presentation function, at least in some cell types. Phosphorylation of the nearby serine residue could potentially modulate recognition of the endocytic motif (as seen for Ii) or it may have a completely separate function. It is clear that much is still unclear regarding the roles of the MHC Class I cytoplasmic domain. The following section will outline the approach used in this thesis work to address some of these questions.
1.5.2 Objectives and general approach

There are two major features that distinguish this thesis work from previous studies on the MHC class I cytoplasmic domain. The first is the creation of class I cytoplasmic tail point mutants that target the highly conserved individual amino acids discussed above. Since previous studies have employed mainly gross cytoplasmic mutations (whole exon deletions and truncations), it has been difficult to learn much about the physiological roles of individual amino acids within the class I cytoplasmic domain. The second unique feature is the expression of these class I point mutants in a transgenic mouse system. Most previous in vitro studies have shown the cytoplasmic region to be dispensable for surface expression and antigen presentation function. However, since the evolutionary pressures that led to the high level of class I cytoplasmic tail conservation took place in vivo, it is perhaps only in an animal model that the true function and significance of the cytoplasmic domain can most clearly be delineated.

The overall objective of this thesis was to utilize both in vitro and in vivo systems to study the effect of specific cytoplasmic mutations on expression, trafficking, and antigen presentation function of H-2 K^b molecules. The in vitro system consisted of murine L cell fibroblasts (H-2^k haplotype) transfected with either the wild-type H-2 K^b gene or one of four cytoplasmic H-2K^b mutant gene constructs (described in Chapter 2). The generation of these transfectants as well as experiments analyzing their class I surface expression, trafficking, and peptide binding are described in Chapter 3.
Transgenic mice expressing the five K\textsuperscript{b} gene constructs (one wild-type and four mutant) were generated, selected and bred onto an H-2\textsuperscript{k} background. Chapter 4 outlines this process in addition to describing experiments performed on lymphocytes derived from the transgenic mice. In particular, detailed studies were made of transgenic and endogenous class I surface expression in peripheral blood leukocytes and splenocytes, and comparisons were made with \textit{de novo} synthesis and maturation rates of both types of molecules. Chapter 4 also describes experiments that characterize the internalization of class I molecules in activated T cells derived from the transgenic mice.

Chapter 5 brings both the \textit{in vitro} and \textit{in vivo} systems together to study antigen presentation. The immunogen used in these studies was the vesicular stomatitis virus (VSV), which generates a well-characterized, immunodominant, K\textsuperscript{b}-restricted epitope in murine systems. The first part of Chapter 5 describes experiments done on the L cell K\textsuperscript{b} transfectants, which were assayed for their abilities to acquire sensitivity to killing by VSV-specific CTL following VSV infection. Acquisition of killing sensitivity is largely indicative of the ability of the K\textsuperscript{b} molecule to acquire and present the VSV-specific N-peptide on the cell surface. The second part of Chapter 5 describes a separate set of VSV experiments that was performed using the K\textsuperscript{b}-expressing transgenic mice. In these experiments, mice were assayed for their abilities to generate specific, K\textsuperscript{b}-restricted CTL in response to VSV infection.
As a conclusion to this thesis, in Chapter 6 I will first summarize the major findings from the work and then set out to propose some models to explain the data presented. The discussion in this section will tend to be more speculative in nature than the discussion sections following each of Chapters 3, 4, and 5. The final part of Chapter 6 will endeavor to point the way to future experimental directions that will build upon the research described in this thesis and may, with any luck, help to uncover some of the remaining mysteries of MHC Class I.
Chapter 2 - Site-directed Mutagenesis of the murine H-2K\textsuperscript{b} Gene

2.1 Cytoplasmic Domain Mutational Strategy

The murine H-2 K gene, allele “b” (designated K\textsuperscript{b}), was modified in four different ways. In order to assess the function of the naturally-occurring Class I exon VII splicing variant, construction of mutant ΔVII was undertaken. This mutant contained a deletion of the entire exon VII coding region and thus coded for a cytoplasmic region 13 amino acids shorter than wild-type K\textsuperscript{b} that lacked the potential serine phosphorylation sites (see Figure 2-1). Another construct, ΔSTSS, was made containing point mutations that altered all three conserved exon VII serine residues as well as a potentially phosphorylatable adjacent threonine residue. This K\textsuperscript{b} variant was constructed to explore the function of MHC Class I phosphorylation in more detail and to better understand the cellular processes mediated by these conserved serine residues. In this construct, all three serine residues were replaced by alanines and the threonine was replaced by isoleucine. Mutant ΔY was generated to address the role of the conserved tyrosine residue in exon VI, as well as to explore its suspected role as part of a tyrosine-based endocytic motif. In this construct, the tyrosine codon was changed to one coding for phenylalanine. The fourth mutant construct, ΔYSTSS, contained point mutations that altered the exon VI tyrosine as well as the exon VII serines and threonine (Figure 2-1).
Figure 2-1: H-2K\textsuperscript{b} cytoplasmic tail mutant constructs.

\textit{(Next page) (top)} Schematic diagram showing intron/exon structure of the H-2 K\textsuperscript{b} gene. The last three exons (VI, VII, and VIII) have been expanded to show the wild-type amino acid sequence of the cytoplasmic region of K\textsuperscript{b}. The highly conserved tyrosine, threonine, and serine residues are highlighted in red. \textit{S, signal sequence; TM, transmembrane domain. (bottom)} Predicted amino acid sequences of the cytoplasmic K\textsuperscript{b} mutants generated by PCR-based site-directed mutagenesis (substituted residues are in green).
**H-2K<sup>b</sup> Gene Exon Structure:**

- **Extracellular region**
  - \(S\) α1 α2 α3 TM I₁ I₂ I₃

- **Intracellular domain**

**K<sup>b</sup>WT**

VI VII VIII

**K<sup>b</sup> Cytoplasmic Mutants:**

- **ΔVII**
  - GGKGGDYALAP (deleted region) VMVHDPHSLA

- **ΔSTSS**
  - GGKGGDYALAP GAQIADLALPDCK VMVHDPHSLA

- **ΔY**
  - GGKGGDFALAP GSQTSDSLDPDCK VMVHDPHSLA

- **ΔYSTSS**
  - GGKGGDFALAP GAQIADLALPDCK VMVHDPHSLA
All site-directed mutagenesis was performed on the murine H-2 K\textsuperscript{b} gene, originally cloned into the pBR322 plasmid (246) to generate pK\textsuperscript{b}. The decision to mutate the K\textsuperscript{b} gene rather than the cDNA was made to accommodate our intention to use the DNA constructs to generate transgenic mice. It was reasoned that the native gene, with its intron/exon structure and natural promotor elements, would be the preferred vehicle for attaining physiological levels of gene expression in a transgenic system.

### 2.2 PCR-based Site-directed Mutagenesis of the K\textsuperscript{b} gene

#### 2.2.1 Mutagenesis Approach

Mutation of the murine H-2K\textsuperscript{b} gene was performed using the PCR-based site-directed mutagenesis approach first described by Ho et al. (247). The approach utilizes four oligonucleotide primers designed in the following manner (see Figure 2-2): Primers 2 and 3 (the mutagenesis primers) are completely complementary in DNA sequence to each other. They are also complementary to the region of the gene one wishes to mutate, except for nucleotide mismatches comprising the mutations one wishes to introduce. The mismatches are optimally located in the central region of the primers so that complementary sequences on either side of the mismatch (a minimum of 12 nucleotides) will facilitate hybridization to the template gene in a PCR reaction. Primer 1 is designed to be exactly complementary to an upstream region of the gene of interest with its 3' end closest to the site of mutagenesis. Primer 4 is exactly complementary in sequence to a downstream region of the template gene, again with its 3' end closest to the mutagenesis
Figure 2-2: PCR-based site-directed mutagenesis approach.

Four oligonucleotide primers (shown as arrows) are used to introduce specific DNA mutations by means of nucleotide mismatches (red cross-hatches). Two initial PCRs to generate primary fragments "A" and "B" are followed by a combination PCR using these two fragments to generate fragment "C". Restriction enzyme (RE) sites flanking the mutation site are used to subclone the DNA fragment containing the desired mutations.
site. Ideally primers 1 and 4 (the "bookend" primers) encompass unique restriction sites flanking the targeted region in the gene of interest that can be used to subclone the mutagenized DNA fragment. Alternatively, primers 1 and 4 can be designed to introduce restriction sites for the purposes of subcloning.

The first PCR reaction utilizes the template gene and primers 1 and 2 to generate DNA fragment A (Figure 2-2). The second PCR reaction uses primers 3 and 4 to generate fragment B. Note that fragments A and B (the "primary" PCR fragments) contain regions of complementary DNA sequence equal to the lengths of primers 2 and 3. A third PCR reaction that includes fragments A and B in addition to primers 1 and 4 will generate fragment C. This "secondary" fragment consists of double stranded DNA spanning the distance from primer 1 to primer 4 that is identical in sequence to the gene of interest except in the regions where nucleotide mutations have been introduced. This fragment can then be cleaved with the appropriate restriction enzymes and subcloned into a vector of interest or back into the original gene, replacing the wild-type sequence (Figure 2-2).

2.2.2 Oligonucleotide design

Three sets of mutagenesis primers were necessary to introduce the desired mutations into the K^b gene. The first set, ΔY2 and ΔY3, were designed to substitute the conserved tyrosine residue within exon VI with a phenylalanine residue (see Table 2-1 for oligonucleotide sequences and Figure 2-3 for homology map). They were utilized in conjunction with bookend primers E6U1 and E6D4 (homologous to regions upstream and
downstream, respectively, of Exon VI) to generate fragment \( \Delta Y \). The second set of mutagenesis primers, \( \Delta STSS2 \) and \( \Delta STSS3 \), were designed to substitute the three exon VII serine residues with alanines and to replace the threonine residue with isoleucine. These primers in conjunction with bookend primers E7U1 and E7D4 were used to generate fragment \( \Delta STSS \). The deletion of exon VII was achieved using a third set of mutagenesis primers, \( \Delta VII2 \) and \( \Delta VII3 \). These oligos were complementary in half of their sequence to the intron region immediately 3' of exon VII and were complementary in their other half to the intron region immediately 5' of exon VII. Used in conjunction with bookend primers E7UP1 and E7D4, fragment \( \Delta VII \), containing a complete deletion of exon VII, was generated.

The bookend primers were designed to be complementary in sequence to intron sequences flanking exons VI and VII. They were also designed to encompass unique restriction sites (where possible) to facilitate future PCR fragment subcloning. Thus, primers E6U1 and E6D4 span the region containing a unique Bgl II site upstream of exon VI as well as an Nsi I site downstream of exon VI (see Figure 2-3). Likewise, primers E7U1 and E7D4 encompass the same unique Nsi I site (upstream of exon VII) in addition to an Sfu I restriction site downstream of exon VII.
## Table 2-1: Primers used for site-directed mutagenesis.

DNA sequences and reference names of all the oligonucleotide primers used in the PCR-based site-directed mutagenesis of the H-2K<sup>b</sup> gene. Three secondary PCR fragments, ΔY, ΔSTSS, and ΔVII were ultimately generated and subcloned into pK<sup>b</sup> to create mutant K<sup>b</sup> constructs ΔY, ΔYSTSS, ΔSTSS, and ΔVII.
Figure 2-3: Oligonucleotide primers mapped to the H-2 K^b gene. The last four exons of the K^b gene are schematized as boxes connected by lines representing intron sequences. Oligonucleotide primers used to introduce specific mutations are aligned to indicate their homology to the K^b gene and are depicted as arrows with the arrowheads representing the 3' ends. Cross-hatches on the primer arrows indicate nucleotide mismatches which give rise to specific cytoplasmic domain point mutations. The dotted lines in primers ΔVII2 and ΔVII3 represent the coding region deleted by those primers, whose sequences are homologous only to the exon VII flanking regions. Pertinent restriction sites used in the cloning procedures are shown in red.
2.2.3 Generation of primary PCR fragments

Successful generation of all primary PCR fragments was performed with PCR conditions consisting of 30 cycles of denaturation at 95°C for 30 s followed by annealing and extension at 60°C for 60 s using Pfu polymerase (Stratagene) in the manufacturer’s buffer and 200 μM dNTPs. The template DNA consisted of a linear Eco RI fragment containing the K<sup>b</sup> gene which had been excised from the pK<sup>b</sup> plasmid and gel-purified (Qiaex II kit). PCR reactions used 10 ng to 50 ng of template, with primer concentrations varying from 100 nM to 500 nM depending on the individual fragment being generated. All reactions were performed using a GeneAmp 9000 PCR machine in 50 μl total volume per tube. To assess the PCR, 15 μl of this volume was analyzed by electrophoresis on a 2% agarose gel containing 200 ng/ml ethidium bromide (EtBr). DNA fragments were visualized using ultraviolet light. The predicted sizes for each of the primary fragments are listed in Table 2-2. Gel photographs for each fragment are shown in Figure 2-4.
<table>
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<th>Primers Used for PCR</th>
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<th>Predicted Size (bp)</th>
<th>Secondary Fragment Name</th>
<th>Predicted Size (bp)</th>
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<tr>
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<td></td>
<td></td>
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<tr>
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<td>ΔY-3/4</td>
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<td></td>
<td></td>
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<td></td>
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<td></td>
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<tr>
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<td>ΔSTSS</td>
<td>424</td>
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<tr>
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<td></td>
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<tr>
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<td>ΔSTSS-3/4</td>
<td>292</td>
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<td></td>
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<tr>
<td>E7D4</td>
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<tr>
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<td>ΔVII-3/4</td>
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<tr>
<td>E7D4</td>
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Table 2-2: Predicted sizes of primary and secondary PCR fragments.

Oligonucleotide primers used to generate each of the fragments are listed in the left-hand column. Primary and secondary PCR fragments are listed with their predicted sizes in nucleotide base pairs (bp).
Figure 2-4: Primary and secondary PCR fragment photographs.

(next page) Gel photographs of primary and secondary PCR fragments used in site-directed mutagenesis of the H-2 K\(^b\) gene. Pairs of primary PCR fragments (left) were generated and subsequently combined to make secondary PCR fragments \(\Delta Y\), \(\Delta STSS\), and \(\Delta VII\) (right). PCR products were run on 2\% agarose gels with ethidium bromide and were photographed under UV light. DNA markers seen in the outer lanes are 100 bp ladders.
Primary PCR fragments

- ΔY-1/2
- ΔY-3/4
- ΔSTSS-1/2
- ΔSTSS-3/4
- ΔVII-1/2
- ΔVII-3/4

Secondary PCR fragments

- ΔY fragment
- ΔSTSS fragment
- ΔVII fragment

(bp: base pairs)
2.2.4 Generation of secondary PCR fragments

PCR reactions were performed as described in Section 2.2.3, with the exception being that the secondary fragments were generated using two primary PCR fragments as template DNA. Also, only the bookend primers were included in the reactions generating secondary DNA fragments (see Figure 2-2). Primary fragments were gel-purified and diluted in ddH2O for use as template DNA in PCR reactions at concentrations ranging from 2 nM to 20 nM. Bookend primers were used at a final concentration of 0.25 μM. PCR reactions were assessed by gel electrophoresis as described in Section 2.2.3. Multiple identical parallel reactions were performed and the samples were pooled to achieve a high yield of secondary fragment DNA for subsequent cloning. The predicted sizes for each of the secondary fragments are listed in Table 2-2. Gel photographs for each fragment are shown in Figure 2-4.

2.3 Subcloning Methods

2.3.1 Subcloning Strategy

The overall strategy for constructing the K\(^b\) cytoplasmic mutants was to replace wild-type sequences in the K\(^b\) gene with PCR-generated DNA fragments that were identical except for the mutations introduced (illustrated in Figure 2-5). This strategy relied on the fact that there are unique restriction sites naturally present in the K\(^b\) intron sequences adjacent to exons VI and VII. Bgl II and Nsi I are the unique cutters flanking exon VI that were
Figure 2-5: H-2K\textsuperscript{b} mutagenesis subcloning strategy.

(next page) The pK\textsuperscript{b} plasmid contains the H-2 K\textsuperscript{b} gene as an Eco RI insert in a pBR322 cloning vector (top). K\textsuperscript{b} exons are depicted as boxes. Wild-type Exon VI was excised from the K\textsuperscript{b} gene by digestion with restriction enzymes Bgl II and Nsi I (left). This was followed by ligation to a Bgl II/Nsi I-cut ΔY fragment to create the ΔY construct (bottom). Exon VII was excised by an Nsi I restriction digest and an Sfu I partial digest (indicated by *, right). This sequence was replaced by either an Nsi I/Sfu I-cut ΔSTSS fragment (to generate the ΔSTSS construct) or ΔVII fragment (to create the ΔVII construct). The ΔYSTSS construct was generated in exactly the same fashion as the ΔY construct, with the exception that the ΔSTSS construct (previously generated) was used in place of the wild-type pK\textsuperscript{b} gene as the cloning vector.
utilized to excise the wild-type exon VI sequence from pK\textsuperscript{b}. The same restriction enzymes were used in parallel to cut the PCR-generated fragment ΔY containing the exon VI tyrosine codon mutation. A ligation of these two fragments resulted in the desired construct ΔY, which was identical to pK\textsuperscript{b} except for the tyrosine substitution.

Constructs ΔSTSS and ΔVII were generated in a similar manner except restriction sites flanking exon VII were utilized. Nsi I was used for the upstream site but there are no unique restriction sites present downstream of exon VII. Sfu I, which cuts three times in pK\textsuperscript{b} including once just 3' of exon VII, was chosen as the best possible enzyme for subcloning the PCR fragments. Thus it was necessary to perform a partial digest of pK\textsuperscript{b} with Sfu I in addition to a complete digest with Nsi I in order to remove and replace the wild-type exon VII sequences. The same two enzymes were also used to cut PCR fragments ΔSTSS and ΔVII, which were subsequently ligated to digested pK\textsuperscript{b} to create constructs ΔSTSS and ΔVII. The ΔYSTSS construct was generated in exactly the same manner as the ΔY construct, with the exception that the ΔSTSS construct (previously generated) was used in place of the wild-type K\textsuperscript{b} gene as the cloning vector for the PCR fragment containing the tyrosine codon mutation (see Figure 2-5).

2.3.2 Restriction Digests of PCR fragments

Each of the three secondary PCR fragments were digested with two different restriction enzymes to facilitate directed cloning into the pK\textsuperscript{b} vector. Fragment ΔY was digested
Figure 2-6: Restriction digests of PCR fragments.

The ΔY PCR fragment was digested with Bgl II and Nsi I to generate a subclonable DNA fragment of 217 bp. ΔSTSS and ΔVII PCR fragments were digested with Nsi I and Sfu I in order to generate subcloning fragments of 159 bp and 114 bp, respectively. Digests were run on 2% agarose gels containing ethidium bromide and were photographed under UV light. Bands (indicated by *) were excised and the DNA was extracted for use in subsequent ligations.
with Bgl II and Nsi I to yield a clonable fragment of 217 bp. Fragments ΔSTSS and ΔVII were digested with Nsi I and Sfu I to yield clonable fragments of 159 bp and 114 bp, respectively (see Figure 2-6). The DNA digests were run on a 2.2% agarose gel and the appropriately sized bands were excised and gel purified (Qiaex II). All restriction enzymes were purchased from Boeringer-Mannheim and all digestions were done at 37°C for three hours in the manufacturer’s buffer “H” using 5 units of enzyme per microgram of DNA.

2.3.3 Restriction Digests of pKb

pKb is a 14.4 kilobase pair (kbp) plasmid consisting of an Eco RI fragment of ~10 kbp containing the H-2 Kb gene cloned into a pBR322 bacterial vector containing an ampicillin resistance marker (see Figure 2-7A). Of the total ~10 kbp insert, only two clusters of 1594 bp and 1837 bp (containing Exons I-III and IV-VIII) have published sequences. Between the two clusters there is ~1200 bp of unknown sequence, and upstream and downstream of the coding regions there is another ~4000 bp and ~1000 bp, respectively, of unknown sequence.

Restriction analysis of the known sequences determined that Bgl II and Nsi I were unique cutters which flanked exon VI. Test digests of pKb confirmed that these sites also were unique to the entire plasmid. This made for a relatively straightforward double digest with these two enzymes in order to excise the wild-type Exon VI coding sequence from pKb. Restriction digests were performed as described in Section 2.3.2. The digested
Figure 2-7A: Schematized restriction map of pK\textsuperscript{b}.

Exons I through VIII are depicted as boxes (along with their encoded protein domain names), and pertinent restriction sites as cross-hatches. Since complete sequences upstream of exon I (SS), exon VI (α3), and downstream of exon VIII (I\textsubscript{3}) are unpublished or unknown, two of the three Sfu I sites indicated are estimated locations (*). Map is not drawn to scale.
Figure 2-7B, C, D: Restriction digests of pK\textsuperscript{b}.

(B) Gel photographs showing restriction digests of pK\textsuperscript{b}. Lane 1, \textit{\Lambda}/Hind III marker; lane 2, uncut K\textsuperscript{b} plasmid; lane 3, Nsi I digest; lane 4, Bgl I digest; lane 5, Nsi I/Bgl II double digest; lane 6, Eco RI digest. (C) Partial Sfu I digest time course. pK\textsuperscript{b} was cleaved first with Nsi I, then with Sfu I for the time points indicated (in minutes). (D) Upscaled Sfu I partial digest. Nsi I-cleaved pK\textsuperscript{b} was digested with Sfu I for 9 minutes and run on an agarose gel. The top band was excised and the DNA was extracted for use in subsequent ligations. All gels shown are 1% agarose with ethidium bromide. DNA standards in the left-hand lanes are \textit{\Lambda}/Hind III markers with fragment sizes as indicated. Asterisks (*) indicate DNA fragments that resulted from incomplete Nsi I digestion.
plasmid DNA was run on a 1% agarose gel (Figure 2-7B) and the appropriate band was excised and gel-purified before ligation to fragment ΔY.

Cutting pKb upstream and downstream of Exon VII proved to be more challenging, mostly due to the fact that the downstream region contains no known unique restriction sites. Sfu I, chosen for the downstream site, was found to cleave pKb into three fragments. It was therefore necessary to perform a partial Sfu I digest of pKb in order to generate the single-cut species needed for cloning purposes. The vector was first linearized by cleaving upstream of exon VII with Nsi I as previously described. Sfu I was then added at 0.5 units of enzyme per microgram of DNA for digestion at 37°C. Aliquots were taken at 6, 12, 20, 30, and 45 minutes and EDTA was added to a final concentration of 10 mM to stop digestion. The samples were run on a 0.8% agarose / EtBr gel and photographed under UV light (see Figure 2-7C). From the results it was apparent that the optimal digestion time for generating the largest amount of single-cut fragment was between 6 and 12 minutes. An larger scale digestion was done using the same conditions but was stopped by addition of EDTA after 9 minutes. The DNA was then run at low voltage on a 0.9% agarose gel overnight to achieve maximal separation and resolution of fragments. The upper band shown in Figure 2-7D was excised and gel purified. This band likely contained a mixture of linearized plasmid (~14400 bp) and Nsi I/Sfu I double-digested pKb (~14240 bp; see predicted fragment sizes in Figure 2-7D).
All gel-purified fragments were treated with one unit of calf alkaline phosphatase (Boeringer Mannheim) per picomole of DNA for 30 minutes at 37°C before extracting with phenol/chloroform and ethanol precipitating the DNA for use in ligations.

2.3.4 Ligations and Bacterial Transformation

Constructs ΔSTSS and ΔVII were generated first by ligating Nsi I/Sfu I doubly-digested pK\textsuperscript{b} to PCR fragments ΔSTSS or ΔVII. Constructs ΔY and ΔYSTSS were subsequently generated by ligating PCR fragment ΔY to either Bgl II/Nsi I-digested pK\textsuperscript{b} or Bgl II/Nsi I-digested ΔSTSS construct (refer to Figure 2-5).

All ligations were done for 16 hours at 18°C using 1 unit T4 DNA ligase (Boeringer Mannheim) in the manufacturer's buffer. Ligations employed an insert to vector molar ratio of 3:1 and a final insert concentration ranging from 1 to 5 ng/µl. All reactions were done in a total volume of 20 µl.

Subcloning efficiency HB101 competent E. Coli (Gibco BRL) were transformed with 2.5 µl of ligation mix by briefly heat-shocking at 37°C followed by incubation in non-selecting LB broth at 37°C for 60 minutes. Bacteria were then grown overnight at 37°C on LB agar plates containing 50 µg/mL ampicillin. Resistant colonies were screened for the presence of the appropriate pK\textsuperscript{b} mutations in a variety of ways that shall be described in the following sections.
2.4 Colony Screening Procedures and DNA Sequencing

2.4.1 Filter Hybridization Screen

Bacterial colonies were streaked onto grid-patterned nitrocellulose filters and grown overnight at 37°C on LB agar plates containing 50 μg/ml ampicillin. Filters were then sequentially placed for 5 minutes each on 3MM paper which was soaked in the following solutions: (1) 1% SDS, (2) 0.5M NaOH / 1.5M NaCl, (3) 1M NH₄Ac / 0.02M NaOH (pH 7.5), (4) 2X SSPE / 0.1% SDS. They were then immersed in chloroform for 45 seconds and blotted with dry 3MM paper. Filters were baked at 80°C in a dry oven to facilitate permanent binding of the bacterial and plasmid DNA to the nitrocellulose membrane.

Three oligonucleotides were designed to probe the plasmid DNA for the presence of the appropriate mutations. All three consisted of shorter versions of the mutagenesis oligos used to create the primary PCR fragments (see Table 2-3). Thus they all contained nucleotide mismatches or deletions (compared to the wild-type Kᵇ sequence) which could be exploited in order to differentiate wild-type from mutant plasmids in a filter hybridization experiment. Mismatches tend to destabilize hybridization and will lower the Tm, or maximum temperature at which hybridization of the oligos can occur.

Oligos (50 ng) were 3' end-labeled for 4 hours with [³²P]-dCTP (1µCi per μl final concentration) using terminal deoxytransferase (Boeringer Mannheim) in the manufacturer's buffer. Unincorporated nucleotides were removed using NickSpin columns. Nitrocellulose filters were soaked in 6X NET solution (Maniatis) for 30
minutes followed by prehybridization at 65°C for 1 hour in 10X Denhardt’s solution / 6X NET / 0.1% SDS. End-labeled probe (1 x 10^7 cpm) was diluted in prehybridization buffer and incubated with the nitrocellulose filters at 42°C overnight. This temperature was chosen based on the predicted Tms of the probe oligos, which were calculated to be in the range of 48-50°C (see Table 2-3). Mismatches between the [^32P]-labeled oligos with mutant sequences and wild-type sequences would decrease the Tm to below 42°C. This would allow differentiation of wild-type and mutant plasmids due to the binding of the probe to mutant, but not wild-type sequences. Filters were washed 3X at 42°C using prehybridization buffer, sealed in plastic wrap and exposed to autoradiograph film. This temperature was effective for distinguishing ΔVII deletion mutants from wild-type K^b plasmids (see Figure 2-8), but could not distinguish ΔY or ΔSTSS mutants. The nitrocellulose filters containing these potential clones were washed again in prehybridization buffer at 65°C, then re-probed overnight at 45°C. At this temperature, ΔSTSS mutants became distinguishable from wild-type plasmids (Figure 2-8). However, repeated probings at 47°, 49°, 50° and 51°C failed to distinguish the ΔY mutation. The probe signal became significantly weaker as the temperature increased but this trend appeared to be homogeneous for all of the bacterial clones. It was therefore necessary to develop a more sensitive method to screen for the 2-nucleotide mismatch present in the ΔY mutant construct.
Figure 2-8: Autoradiographs of filter hybridization screen.

Autoradiographs from ΔVII & ΔSTSS mutant filter screens. Nitrocellulose filters were streaked with ampicillin-resistant *E. Coli* following transformation with DNA ligation mixes, then probed with ³²P-labelled oligonucleotides containing mutant-specific DNA sequences. Positive bacterial clones are indicated with arrows.
2.4.2 PCR-based Differential Temperature Screen

I decided to use a PCR-based method of screening for constructs ΔY and ΔYSTSS, due largely to the fact that the GeneAmp 9000 PCR machine possessed a more sophisticated temperature control (accurate to tenths of degrees Celsius) compared to the water bath used in the filter hybridization screen. Also, it was reasoned that any small differences in probe binding caused by mismatches would be amplified by PCR cycling, making it easier to distinguish wild-type constructs from the tyrosine codon mutants. Oligo ΔYX was specially designed for the PCR-based screen (Table 2-3), to be used in conjunction with oligo E6U1 to generate a 200 bp DNA fragment under full DNA template binding conditions (see Figure 2-9). The 2-nucleotide mismatch comprising the tyrosine codon mutation was designed to be at the 3’ end of the ΔYX primer, making priming of DNA from a wild-type template difficult at temperatures approaching or above the probe’s Tm.

The first step was to empirically derive the set of PCR conditions that would generate a 200 bp fragment using a tyrosine codon-mutated DNA template but not a wild-type K\textsuperscript{b} template. Based on earlier PCR experiments, it was decided to use conditions consisting of 32 cycles of denaturation at 95°C for 30 s followed by annealing and extension for 45 s using Pfu polymerase (Stratagene) in the manufacturer’s buffer and 200 μM dNTPs. Primers E6U1 and ΔYX were used at 400 nM each. DNA templates consisted of the wild-type K\textsuperscript{b} gene (negative control), the ΔY PCR fragment (positive control), and a
PCR-based Differential Temperature Screen

Figure 2-9: PCR-based screening strategy.

PCR screen designed to distinguish wild-type K\textsuperscript{b} plasmids from constructs containing the ΔY mutation. Oligonucleotide primer ΔYX is exactly complementary in sequence to ΔY-containing constructs, but differs from the K\textsuperscript{b} wild-type sequence by two nucleotides at its 3' end (red arrowhead). In a polymerase chain reaction with primer E6U1, this mismatch can be exploited to distinguish DNA constructs that possess the ΔY mutation. At the correct annealing temperature, the two primers will generate a 200 bp product with the ΔY construct as template DNA while no product is generated using wild-type K\textsuperscript{b} as a template.
potential ΔY gene construct which had given a weakly elevated signal upon filter hybridization. The annealing and extension temperature used was 67°C, five degrees below the Tm of the ΔYX oligo. The PCR results showed that a 200 bp fragment was generated with all three templates (Figure 2-10A). However, using an annealing/extension temperature of 74°C no fragments were generated for any of the three templates. A series of PCR reactions ensued, using the same conditions outlined above except for varying the annealing/extension temperatures of the PCR cycles. The temperature at which the ΔY mutation could be distinguished from wild-type sequence was found to be 71.1°C (Figure 2-10A). Under these conditions, a 200 bp fragment was generated using the ΔY fragment as a DNA template but not while using the wild-type Kb gene. The potential ΔY construct also generated a 200 bp fragment, indicating that it too contained the ΔY mutation.

DNA plasmid minipreps (Maniatis) of 20 randomly-chosen clones were performed for each of the two batches containing potential constructs ΔY and ΔYSTSS. The miniprep DNA was cleaved with Eco RI to linearize the Kb gene and half of each sample was run on a 1% agarose gel to confirm its normal Eco RI restriction pattern. The remainder of the sample was used as template DNA for the PCR-based screen described above. The screen was able to clearly distinguish between clones containing wild-type sequences and those containing the ΔY mutation (Figure 2-10B). Suspected point mutations were later confirmed by DNA sequencing.
<table>
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<td>48</td>
<td>filter hybridization probe</td>
</tr>
<tr>
<td>ΔY2.1</td>
<td>5'&lt;CCAGAGGCTCCC&gt;3'</td>
<td>50</td>
<td>filter hybridization probe</td>
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<tr>
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<td>72</td>
<td>primer for PCR-based screen</td>
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</table>

Table 2-3: Oligonucleotides used for screening mutant clones.

Listed are the reference name, DNA sequence, predicted hybridization melting temperature (Tm), and screening function for each of the oligos.
Figure 2-10: Results of PCR-based differential temperature screen.

PCRs were performed at the various annealing temperatures indicated in order to empirically determine the temperature at which the ΔY mutation (a 2 nt mismatch) could be distinguished from the K^b wild-type sequence. Lanes 1-7 differ only in the template DNA used in the PCR: Lanes 1 & 2: linearized pK^b (wild-type control); Lanes 3 & 4: ΔY PCR fragment (mutant control); Lanes 5 & 6: potential ΔY construct (experimental sample); Lane 7: no template DNA (negative control). (B) Results of miniprep screen. ΔY and ΔYSTSS constructs are clearly distinguishable from wild-type K^b sequences using the PCR-based screen at an annealing temperature of 71.1°C.
2.4.3 DNA sequencing

The Nucleic Acid-Protein Sequencing (NAPS) Unit at the University of British Columbia, Vancouver, Canada, performed DNA sequencing of DNA derived from screened bacterial clones. They were provided with 1 µg of plasmid DNA and 100 ng of each of the bookend primers E6U1, E6D4, E7U1, and E7D4. Sequencing was done in both directions from the primers listed and 450-650 bp of usable sequence was typically obtained. The validity of all four mutant K\textsuperscript{b} constructs was confirmed by analysis of the sequencing data provided.

2.4.4 Maxiprep and Cesium Chloride Purification of Plasmids

A large scale plasmid prep (Maniatis) was performed for pK\textsuperscript{b} and each of the four mutant constructs ΔY, ΔVI, ΔSTSS, and ΔYSTSS. The plasmid DNA was further purified by running the sample on a cesium chloride gradient. The DNA band was identified by UV light and extracted from the gradient using a 22 gauge syringe needle. Ethidium bromide was removed by repeated extractions of the DNA samples with isoamyl alcohol. Cesium chloride was removed by dialyzing the sample overnight in TE buffer. Samples were ethanol precipitated and resuspended in TE buffer, pH 8. Purity and concentration were assessed by spectrophotometry as well as by gel electrophoresis. This circular plasmid was used as the DNA source for all L cell fibroblast transfections (Chapter 3). For the purpose of microinjections, the pBR322 plasmid sequences were removed from the gene constructs because plasmid sequences are known to repress transgene expression. Purified plasmid DNA was cleaved with Eco RI and the DNA fragments were separated.
on a 1% agarose gel. The ~10 kbp band (containing the K\textsuperscript{b} gene) was excised from the gel and purified by Qiaex II. The DNA was subsequently diluted to 1 µg/ml for microinjection into fertilized murine oocytes (Chapter 4).
Chapter 3 - Transfection and Analysis of L Cell Fibroblasts (K<sup>b</sup> Expression and Trafficking)

3.1 Introduction

A great deal has been learned about MHC class I structure, oligomerization and function by studying these molecules in transfected cell lines. Most of the studies designed to elucidate the function of the class I intracellular domain have also been conducted in these systems. It was first reported in 1983 that class I molecules containing cytoplasmic truncations could still be expressed at the plasma membrane in a variety of cell types. Truncated class I molecules were reported to undergo intracellular transport and maturation at very similar rates to wild-type molecules (214, 215). Furthermore, the truncated Class I molecules were also reported (for the most part) to function capably as targets for both allogeneic and peptide-specific CTL (more on this in Chapter 5). These studies, combined with several ground-breaking reports concerning peptide-binding and T cell receptor/CD4/CD8 recognition of the Class I extracellular domain, likely served to deflect much attention away from the intracellular region. Consequently, over the past several years, studies on the MHC Class I cytoplasmic domain have been relatively few in number.

The only function that has been definitively attributed to the intracellular region is a role in MHC Class I cell surface internalization in activated T cells (see Chapter 4) (188, 208). However, there have been other studies that hint at a broader function for this
region. For example, one study implicated the cytoplasmic domain in intracellular transport by demonstrating that this region is inaccessible to biological probes during its transport from the ER to the cell surface (219). Another report characterized monoclonal antibodies against H-2D\textsuperscript{d}, one of which reacted with an exon VII epitope in non-phosphorylated, non-\(\beta_2\)-microglobulin-bound Class I H chains (248). This form was shown to be antigenically distinct from phosphorylated, \(\beta_2\)-m-bound D\textsuperscript{d}. The result was supported by a previous report that characterized antisera raised against the cytoplasmic exon VI, VII, and VIII regions of H-2K\textsuperscript{b}. This study found that exon VI and exon VII antisera could only immunoprecipitate free, non-\(\beta_2\)-m bound forms of class I, whereas exon VIII antisera was able to recognize both free and \(\beta_2\)-m-bound forms of H-2K\textsuperscript{b}. The authors concluded that \(\beta_2\)-m binding to Class I H chain caused a conformational change not only in the extracellular region, but in the cytoplasmic domain as well (249). According to their model, this conformational change resulted in a lack of recognition of K\textsuperscript{b} by exon VI- and VII-specific antisera, perhaps by masking the epitopes or rendering them inaccessible. Since the exon VII-specific antiserum was generated against a non-phosphorylated peptide, it is possible that serine phosphorylation is the cause of the lack of epitope recognition.

The latter two studies both imply a link between Class I H chain phosphorylation and \(\beta_2\)-m binding. They have led to speculation that a mechanism may exist by which the cell is able to detect "empty" Class I molecules on the cell surface and preferentially internalize them. This mechanism would be driven by phosphorylation and could allow the cell to clear empty MHC complexes, which are not capable of antigen presentation to
T cells, from the cell surface. It is known that functional MHC complexes can be regenerated from empty surface Class I H chains and exogenously-added β2m and specific peptide (250, 251). It is therefore probably in the best interest of the cell to internalize empty H chains in order to prevent the indiscriminate presentation of exogenously-derived antigens which may target the cell for killing by CTL.

Since cytoplasmic determinants have been implicated in the trafficking of several transmembrane proteins, we wanted to determine whether MHC class I K\textsuperscript{b} cytoplasmic mutants demonstrated altered expression or oligomerization compared to wild-type K\textsuperscript{b} molecules. This chapter outlines the transfection of murine L cell fibroblasts (H-2\textsuperscript{k} haplotype) with each of the K\textsuperscript{b} constructs described in Chapter 2, and describes experiments performed on the transfectants to study the surface expression, trafficking, peptide binding, and β2m association of Class I molecules. Since cytoplasmic determinants had previously been shown to affect internalization in T cells, we were interested in testing whether cytoplasmic mutations could affect basal levels of Class I surface expression in fibroblasts. Thus, FACScans were performed on all of the L cell transfectants to measure surface expression of K\textsuperscript{b} and of endogenously-encoded K\textsuperscript{k}. To further characterize the K\textsuperscript{b} mutants, \textsuperscript{35}S pulse-chase experiments were done to determine maturation rates and cellular half-lives of the different Class I molecules. Finally, to explore whether phosphorylation is associated with oligomerization of Class I complexes, experiments were performed to compare β2m association and peptide binding between K\textsuperscript{b} wild-type molecules and cytoplasmic K\textsuperscript{b} mutants.
3.2 Materials and Methods

3.2.1 Transfection of L cell fibroblasts

L cell fibroblasts were grown and maintained at 37°C in tissue culture dishes in a humidified incubator with 5% CO2. The tissue culture medium consisted of Dulbecco's Modified Eagle Medium (DMEM) supplemented with 2mM L-glutamine, 10% fetal bovine serum (FBS), and was buffered with 20 mM HEPES. Cells were grown to 50% confluence in 60 mm dishes and washed twice with serum-free DMEM. Cesium chloride-purified pK\textsuperscript{b} plasmid DNA (wild type or mutant) was combined in a 10:1 molar ratio with pSVneo plasmid DNA along with 50 μl dH\textsubscript{2}O and 50 μl lipofectin and allowed to stand at room temperature for 15 minutes. This mixture was then added to each plate of L cells along with 1.5 mL serum-free DMEM. Each 60 mm plate (containing approximately 1.5 x 10\textsuperscript{6} L cells) received either 3 μg or 10 μg of total plasmid DNA. Cells were incubated overnight and the following day 3.0 mL DMEM was added with 0.5 mL FBS. After another overnight incubation the cells were split into two 100 mm dishes and cultured in normal medium supplemented with 500 μg/mL G418 (Figure 3-1). Dead cells were removed and fresh selection media was added every 2 or 3 days. G418-resistant colonies were trypsinized using cloning rings and grown up as clonal populations in 6-well tissue culture dishes.
3.2.2 Screening of Potential Transfectants

G418-resistant clones were screened by surface labeling with a K\textsuperscript{b}-specific monoclonal antibody, followed by FACScan analysis. Briefly, 1x10\textsuperscript{5} to 5x10\textsuperscript{5} adherent cells were treated with Versene to remove them from tissue culture plates, incubated in 100 µl 20.8.4s hybridoma supernatant (K\textsuperscript{b}-specific), washed, and incubated in 100 µl goat anti-mouse IgG conjugated to FITC (1:100 dilution). The cells were then washed extensively, fixed in 1.5% paraformaldehyde and stored in the dark at 4°C until FACScan analysis could be performed. Parallel cultures were maintained and clones showing positive surface labeling for K\textsuperscript{b} were frozen in DMEM with 20% FBS and 10% DMSO and stored in liquid nitrogen. Clones displaying a mixed population of K\textsuperscript{b}-positive and -negative cells underwent further purification steps to isolate the positive fraction. Clones showing 20% or higher K\textsuperscript{b}-positive cells were purified using limiting dilution techniques while clones with less than 20% K\textsuperscript{b}-positive cells were purified by FACSort (Figure 3-1). Using these screening methods, a number of fibroblast clones expressing each K\textsuperscript{b} construct were isolated: K\textsuperscript{b}WT (5 clones), ΔY (5), ΔVII (6), ΔSTSS (8), and ΔYSTSS (3).

3.2.3 Fluorescent labeling of cells

L cell transfectants were analyzed quantitatively for cell surface expression of the transfected MHC Class I allele K\textsuperscript{b}, endogenous alleles K\textsuperscript{k} and D\textsuperscript{k}, and B\textsubscript{2}m using the monoclonal antibodies 20.8.4s and AF6.88.5.3 (both K\textsuperscript{b}-specific), 16.3.1N and 16.1.11N (both K\textsuperscript{k}-specific), 15.5.5s (D\textsuperscript{k}-specific), and a monoclonal antibody specific for β\textsubscript{2}m. Transfected cells were removed from tissue culture dishes using Versene. Cells were
counted and incubated for 30 minutes at 4°C in 100 μl specific hybridoma supernatant previously titrated to optimally saturating conditions for 1x10^6 L cells. Cells were then washed three times and incubated in FITC-conjugated goat anti-mouse IgG for 30 minutes.

Labeled cells were washed again extensively before fixing in 1.5% paraformaldehyde and storing in the dark at 4°C. For serum deprivation and peptide addition experiments, parallel 60 mm tissue culture dishes containing fibroblasts grown to 50% confluence were used. Plates of cells were either grown for 24 hours prior to FACS staining in serum-free DMEM or complete DMEM. To each group of plates VSV N-peptide (52-59) (RGYVYQGL) was added in the media at concentrations of 0 nM, 200 nM, or 1 μM for 24 hours prior to staining. Ovalbumin peptide addition experiments were performed by adding 1 μM OVA peptide (SIINFEKL) to the cells 3 hours prior to FACS staining.
Figure 3-1: Fibroblast transfection and screening summary.

(next page) L cells were cotransfected with the pSVneo plasmid and one of the five \( K^b \) constructs. G418-resistant colonies were isolated, grown and stained for \( K^b \) surface expression. Mixed populations were purified either by FACSsort or limiting dilution, depending on how prevalent the \( K^b \)-positive cells were within the population. This protocol resulted in 5 \( K^b \text{WT} \)-expressing clones, 8 \( \Delta \text{STSS} \) clones, 6 \( \Delta \text{VII} \) clones, 3 \( \Delta \text{YSTSS} \) clones, and 5 \( \Delta \text{Y} \) clones.
Co-transfect L cell fibroblasts
Select colonies for G418 resistance
FACScan ~180 resistant clones for $K^b$ surface expression

Mixed populations

- $K^b$ negative (~150 clones)
- $<20\% K^b$ positive
- $>20\% K^b$ positive

K$^b$ positive clones:
- 5 $K^b$ WT
- 8 $\Delta$STSS
- 6 $\Delta$VII
- 3 $\Delta$YSTSS
- 5 $\Delta$Y
3.2.4 Pulse-chase Experiments

L cell transfectants grown to 80% confluence on 60 mm dishes were starved in methionine and cysteine-free media for 20 minutes, pulsed with $[^{35}\text{S}]$cysteine/methionine (200 $\mu$Ci/mL) for 20 minutes, and chased in medium containing excess methionine and cysteine (0.5 mM of each). At the indicated time points, plates were washed with ice cold phosphate-buffered saline (PBS) and cells were lysed in 1% Nonidet P-40 (NP-40) buffer (120 mM NaCl, 4 mM MgCl$_2$, 20 mM Tris-HCl pH 7.6) containing 40 $\mu$g/mL PMSF for 20 min at 4°C. After cell nuclei had been centrifuged for 15 minutes, cell lysates were TCA-precipitated and equal numbers of counts were precleared with normal rabbit serum and Protein A-sepharose (Pharmacia) prior to immunoprecipitation with mAbs 20.8.4s or 16.3.1N. Thereafter, protein A-Sepharose beads were added to bind immune complexes. The beads were washed four times and were boiled in 30 $\mu$l of 3.5 % SDS / 30% sucrose / 80 mM Tris (pH 8.8) / 0.01% bromophenol blue containing 20 mM dithiothreitol for 5 min before adding iodoacetamide to 200mM and separating proteins on a 10-15% gradient gel by SDS polyacrylamide gel electrophoresis (PAGE). The gels were fixed, enhanced, dried and exposed to autoradiographic film.

3.2.5 Densitometry

Autoradiograph gel band intensities were quantitatively analyzed by densitometry using an Alpha Imager 2000 Documentation and Analysis System. Gels were imaged in a Multi Image Light Cabinet and analyzed by AlphaEase Version 4.0 computer imaging software.
(Alpha Innotech Corporation). Gel band intensity peaks were generated by scanning of the bands. These peak curves were subsequently integrated to give quantitative comparative intensity ratios for two or more gel bands.

3.3 Results

3.3.1 Surface Expression of H-2 $K^b$, $K^k$, and $\beta_2$-microglobulin

Since cytoplasmic determinants had previously been implicated in the endocytosis of class I molecules from the cell surface in T cells, we wanted to explore whether the $K^b$ mutations would alter the steady-state levels of surface class I in fibroblasts. L cell transfectants were quantitatively stained with $K^b$, $K^k$, and $D^k$-specific antibodies and analyzed by FACScan for surface expression levels of these MHC class I alleles. The results of this staining are shown in Figure 3-2, with $K^b$ surface expression (x-axis) plotted against $K^k$ surface expression (y-axis). While the results at first glance appear to be quite scattered, upon closer inspection a general trend can be observed. With respect to $K^b$ expression, the $\Delta$VII mutant clones showed somewhat higher surface levels and also a greater range of expression levels. Average fluorescence levels for each group of clones were: $K^b$WT (131 ± 38 AFU), $\Delta$Y (194 ± 83 AFU), $\Delta$VII (253 ± 95 AFU), $\Delta$STSS (168 ± 88 AFU), and $\Delta$YSTSS (143 ± 56 AFU).

Perhaps more striking was the effect that $K^b$ gene expression seemed to have on endogenous (ie. $K^k$ and $D^k$) class I surface expression. Compared to untransfected L cells,
transfectants expressing the wild-type K<sup>b</sup> gene showed reduced K<sup>k</sup> and D<sup>k</sup> surface levels which correlated well with increasing K<sup>b</sup> surface levels (Figure 3-2). In contrast, the majority of the L cells (22 out 26) expressing mutated K<sup>b</sup> genes showed little or no reduction in endogenous class I levels, despite the fact that K<sup>b</sup> surface expression reached significantly higher levels. Curiously, for seven of the mutant clones, K<sup>k</sup> levels were significantly increased (29% ± 6.5%, p=0.05) compared to untransfected controls. Both endogenous class I alleles K<sup>k</sup> and D<sup>k</sup> were affected similarly by K<sup>b</sup> gene expression (data not shown).

In addition to staining the L cells for Class I surface expression, β<sub>2</sub>-microglobulin (β<sub>2m</sub>) surface levels were also examined. Two clones from each group (representing low and high K<sup>b</sup> expressors) were stained with anti-class I in addition to anti-β<sub>2m</sub> monoclonal antibodies. The results, summarized in Table 3-1, indicated a subtle yet significant difference (p=0.05) in β<sub>2m</sub> surface levels between fibroblast clones expressing only wild-type class I and those expressing mutant class I molecules. For untransfected L cells and L cells transfected with the K<sup>b</sup>WT gene, the mean ratio of β<sub>2m</sub> expression to total class I expression was calculated to be 0.72 ± 0.03. In the L cell clones expressing mutant K<sup>b</sup>, this ratio was significantly lower, an average of 0.53 ± 0.06. All numbers shown in Table 3-1 denote the means of triplicate FACScan experiments. Ratios were calculated by dividing β<sub>2m</sub> surface fluorescence by the sum of individual surface fluorescences measured for class I alleles K<sup>b</sup>, K<sup>k</sup> and D<sup>k</sup>. 

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Figure 3-2: MHC Class I surface expression in transfected murine fibroblasts.

L cell transfectants were stained for surface expression of H-2K^b or endogenously-expressed K^k and quantitatively analyzed by FACScan. All transfectants contained two copies of the K^k gene and variable numbers of copies of the transfected K^b gene. Untransfected L cells are plotted on the y-axis for comparison to normal levels of K^k surface expression. Trendlines are indicated for K^bWT-expressing clones as well as for each of the four mutant groups.
3.3.2 Effects of Serum and Peptide on Class I Surface Expression

In addition to the Class I heavy chain, both β2m and peptide are required to form a complete MHC Class I complex. Occasional dissociation of these complexes is thought to occur on the cell surface and phosphorylation has been postulated as the mechanism by which these empty heavy chains are identified and removed from the cell surface. Since complete Class I complexes can be reconstituted by adding exogenous β2m and specific peptide to surface-bound heavy chain, we wanted to test whether any differences in MHC complex reconstitution could be observed between KbWT molecules and Kb cytoplasmic mutants.

Fetal bovine serum (FBS) is known to contain bovine β2m, which can bind to murine Class I heavy chains. Serum deprivation, therefore, removes the exogenous β2m source that is present as part of normal cell culture conditions. We wanted to test whether serum deprivation affected wild-type and cytoplasmic mutant Class I molecules differently. The effect of the other variable, peptide, was tested by the addition of two exogenous Kb-specific peptides. The results of a representative experiment are summarized in Figure 3-3. Fibroblasts incubated for 24 hours in serum-free media showed an average reduction in cell surface expression of conformationally-dependent Kb epitopes of 30.4 ± 3.3% (n=3) compared with cells grown in FBS. Both KbWT- and ΔY mutant-expressing clones showed similar levels of reduction (~25%), while ΔVII, ΔSTSS, and ΔYSTSS showed moderately higher reduction in surface Kb complexes (~35%). Serum deprivation reduced
Table 3-1: MHC Class I and β₂-m surface expression in transfected fibroblasts.

Comparison of total MHC class I surface expression and β₂-microglobulin surface expression in selected L cell transfectants. Two clones (low and high K<sup>b</sup> expressors) from each of the L cell transfectant groups, as well as two untransfected L cell clones, were stained for surface expression of K<sup>b</sup>, K<sup>k</sup>, D<sup>k</sup>, and β<sub>2</sub>m. Cells were quantitatively analyzed by FACScan and the mean fluorescences in AFU are listed in the table above. The right column shows the ratio of β<sub>2</sub>m surface expression to total MHC class I surface expression (sum of the AFUs for each of the three alleles).
conformationally-dependent surface K^k levels by a similar extent in all clones (average reduction of 23.6 ± 5.7% (n=2), data not shown).

The addition of exogenous VSV N-peptide (52-59) revealed a small but reproducible difference in the ability of K^b WT and K^b mutant clones to reconstitute conformational K^b complexes. Three separate K^b WT-expressing clones failed to show a significant increase in surface-bound K^b complexes after a 24 hour incubation with 1 μM VSV N-peptide, regardless of the presence of FBS (average increase: 2.1 ± 0.7%, n=3). However, eight mutant clones demonstrated an average increase of 12.3 ± 2.9% (n=3) under the same peptide-binding conditions, also regardless of the presence of serum. All four K^b mutants showed similar increases in surface expression of the conformational epitope compared to the wild-type clones.

A variation of this experiment was performed using OVA peptide and a monoclonal antibody that is specific for K^b-OVA peptide complexes (25.D1) (252). The results, summarized in Figure 3-4, clearly demonstrated that L cells expressing cytoplasmically mutated K^b have a higher capacity for binding exogenously-added OVA peptide than do K^b WT-expressing cells. One low- and one high-expressing clone from each K^b transfectants group was pulsed with 1 μM OVA peptide for 3 hours and subsequently stained with 25.D1 prior to FACScan analysis. All clones not pulsed with peptide showed a similarly low level of staining with the K^b-OVA specific antibody. However, while
Figure 3-3: Effects of serum and VSV peptide addition on $K^b$ surface expression.

L cell transfectants were either cultured for 24 hours in serum-free medium, with or without 1 uM VSV-N peptide (52-59), or were cultured in 10% fetal bovine serum (FBS) with or without 1 uM VSV N peptide. Cells were then stained and quantitatively analyzed for $K^b$ surface expression using FACScan.
peptide-pulsed K\textsuperscript{b}WT clones showed an average increase of only 4 AFU, ΔY clones increased 8.5 AFU, ΔSTSS clones 10.5 AFU, ΔVII clones 23 AFU, and ΔYSTSS clones 27.5 AFU. There was a correlation observed between the overall level of K\textsuperscript{b} expression and the increase in surface fluorescence due to K\textsuperscript{b}-OVA-specific staining following peptide addition. However, comparison of K\textsuperscript{b}WT and K\textsuperscript{b} mutant clones expressing similar levels of K\textsuperscript{b} demonstrated that mutant clones consistently bound 2 to 4 times more OVA peptide than wild-type clones. ΔVII and ΔYSTSS clones bound the most peptide, with ΔY and ΔSTSS clones demonstrating a more modest peptide-binding ability.
Figure 3-4: Effect of OVA peptide addition on K<sup>b</sup> surface expression.

Two clones from each L cell transfectant group (low and high K<sup>b</sup> expressors, see Table 3-1) were pulsed for 3 hours with 1 μM OVA peptide (SIINFEKL), stained with a K<sup>b</sup>-OVA peptide complex-specific monoclonal antibody (25.D1), and analyzed by FACScan.
3.3.3 SDS-PAGE analysis of H-2 \( K^b \) and \( K^k \)

In order to characterize the \( K^b \) mutants further, an \(^{35}S\) labeling of fibroblast clones was performed. Cells were pulsed for 20 minutes and chased for two hours before immunoprecipitating with \( K^b \)- or \( K^k \)-specific mAbs. The proteins were run on SDS-PAGE and the gel was exposed to autoradiographic film. Figure 3-5 shows that the \( K^b \) protein immunoprecipitated from \( K^b \) WT transfectants ran at an apparent MW of \(-45 \) kD. \( \Delta Y K^b \) ran at a MW indistinguishable from \( K^b \) WT. \( \Delta VII-K^b \), containing a deletion of 13 amino acids, ran as expected at a lower apparent MW of \(-43 \) kD. Surprisingly, \( \Delta STSS-K^b \) ran at an apparent MW of \(-43.5 \) kD and \( \Delta YSTSS-K^b \) at a slightly lower \(-42.5 \) kD. \( K^k \) immunoprecipitated from all transfectants ran at an apparent MW of \(-44.5 \) kD.

3.3.4 Pulse-chase experiments

L cell transfectants were analyzed by pulse-chase techniques in order to compare the maturation rates and the half-lives of the \( K^b \) mutants to their wild-type counterparts. Parallel plates of fibroblasts were pulsed for 20 minutes with \(^{35}S\)-cysteine/methionine and then chased for various times before lysing the cells and immunoprecipitating with \( K^b \)- or \( K^k \)-specific mAbs. Results are shown in Figures 3-6 and 3-7. Maturation of Class I molecules was monitored by their acquisition of Endo H resistance over time. This provided a measure of the transport rate of the molecules as they progressed from the endoplasmic reticulum (ER) to the cell surface. Figure 3-6 shows that in cell lines expressing comparable levels of surface \( K^b \), the rates of Endo H resistance acquisition were very similar for \( K^b \) WT and \( K^b \) mutant molecules, with mutant molecules maturing
slightly more slowly. Maturation rates were generally slower all for clones expressing high surface levels of K\(^b\) (see Figure 3-6, ΔSTSS sample). Likewise, the half-lives of the conformational epitopes were determined by the rate of disappearance of the radioactive gel band signal over time. A slightly shorter half-life was observed for K\(^b\)WT molecules in some experiments, but this was not seen consistently enough to be considered statistically significant. Overall, the results suggest that K\(^b\)WT and K\(^b\) mutant molecules have very similar conformational half-lives in L cell fibroblasts.
Figure 3-5: SDS-PAGE size comparison of K\textsuperscript{b} mutants.

Five L cell transfectants expressing comparable levels of cell surface K\textsuperscript{b} were pulsed with \[^{35}\text{S}\] cysteine/methionine for 20 minutes and chased for two hours before lysing the cells in 1% NP-40 and immunoprecipitating equal TCA-precipitable counts with either K\textsuperscript{b}- or K\textsuperscript{k}-specific monoclonal antibodies. Samples were run on a 10-15% SDS-PAGE gradient gel and visualized by autoradiography. The K\textsuperscript{b} genes expressed by each clone are: lane 1, K\textsuperscript{b} wild-type; lane 2, \(\Delta Y\); lane 3, \(\Delta V I I\); lane 4, \(\Delta S T S S\); lane 5, \(\Delta Y S T S S\). MW: \[^{14}\text{C}\] labelled molecular weight marker (43 kD).
Figure 3-6: L cell pulse-chases (+/- Endo H).

Pulse-chases of transfected L cell fibroblasts showing acquisition of Endo H resistance. Fibroblasts transfected with indicated K\textsuperscript{b} constructs were pulse-labeled with \[^{35}\text{S}]\) cysteine/methionine for 20 minutes and chased for various times before immunoprecipitation with the K\textsuperscript{b}-specific monoclonal antibody 20.8.4s. IPs were digested with Endoglycosidase H (or not), run on SDS-PAGE, and exposed to autoradiographic film. Lanes 1-6: Indicates chase times of 0, 30 minutes, and 1, 2, 4, and 8 hours, respectively.
Figure 3-7: Pulse-chases of transfected L cell fibroblasts.

Fibroblasts transfected with indicated K\(^b\) constructs were pulse-labeled with \(^{35}\text{S}\) cysteine/methionine for 20 minutes and chased for various times before immunoprecipitation with the K\(^b\)-specific monoclonal antibody 20.8.4s. IPs were run on SDS-PAGE and exposed to autoradiographic film. Lanes 1-8: Indicates chase times of 0, 30 minutes, and 1, 2, 3, 4, 6, and 8 hours, respectively.
3.3.5 *Comparison of protein synthesis and surface expression*

The most consistent difference observed between K<sup>b</sup>WT and K<sup>b</sup> mutant molecules in the [³⁵S] experiments was their extent of radioactive labelling. Equal numbers of L cell transfectants expressing similar levels of surface K<sup>b</sup> and K<sup>k</sup> (as determined by FACScan) were labelled for identical periods of time. Protein labelling was quantified by counting cell lysate TCA-precipitable radioactivity. Equal counts were immunoprecipitated with K<sup>b</sup>- or K<sup>k</sup>-specific mAbs and run on SDS-PAGE. Despite similar levels of overall [³⁵S] labelling and K<sup>b</sup> surface expression, K<sup>b</sup> mutant clones consistently labelled fewer K<sup>b</sup> molecules than K<sup>b</sup>WT clones, as measured by the density of the radioactive gel bands. Figure 3-8 shows results from three separate experiments. One of these compared a K<sup>b</sup>WT clone and AYSTSS clone expressing nearly identical K<sup>b</sup> surface levels. Despite similar labelling treatments, the AYSTSS clone labelled 60% less K<sup>b</sup> than the K<sup>b</sup>WT clone. Figure 3-8 shows that K<sup>k</sup> molecules, immunoprecipitated as a control, were labelled at a comparable level in both fibroblast clones. Even mutant clones demonstrating significantly higher K<sup>b</sup> surface expression showed less radioactive labelling than wild-type K<sup>b</sup> (Figure 3-8). This trend was consistently observed over several experiments (see Figure 3-5 and 3-9, for example) and was noted particularly for the ΔVII, ΔSTSS, and ΔYSTSS mutants. This result may indicate that the ratio of K<sup>b</sup> synthesis rate to surface expression level may differ between wild-type and mutant K<sup>b</sup> molecules.
Figure 3-8: Comparison of Class I synthesis and cell surface expression.

Equal numbers of transfected L cell fibroblasts were pulse-labeled with \(^{\text{[35S]}}\) cysteine/methionine for 60 minutes and chased for two hours before lysis and immunoprecipitation of equal TCA-precipitable counts with the \(K^b\)-specific monoclonal antibody 20.8.4s or the \(K^k\)-specific mAb 16.3.1N. IPs were run on SDS-PAGE and exposed to autoradiographic film. Parallel unlabeled samples were analyzed for class I surface expression by FACScan using the same two mAbs. Numbers indicate mean fluorescence in arbitrary fluorescence units (AFU). Three separate experiments are shown: Lanes 1 & 2: \(K^b\)WT clone and ΔVII clone; Lanes 3 & 4: \(K^b\)WT clone and ΔYSTSS clone; Lanes 5 & 6: \(K^b\)WT clone and ΔSTSS clone. Similar results were observed using two other conformation-specific mAbs (data not shown).
3.3.6 Quantitation of MHC Class I / β2m Association by Densitometry

β2m can be co-immunoprecipitated with Class I molecules using Class I-specific antibodies (Figure 3-9). Therefore, to test whether cytoplasmic mutations affect the ability of K\(^b\) molecules to associate with β2m during biosynthesis and surface expression, densitometry experiments were performed on \(^{35}\text{S}\) pulse chase autoradiographs in order to quantify the K\(^b\)/ β2m gel band intensity ratios. Measurements from numerous autoradiographs showed that wild-type K\(^b\) molecules, mutant K\(^b\) molecules, and K\(^k\) molecules were all associated with β2m at similar gel band intensity ratios at all experimental time points (data not shown). This result demonstrates that K\(^b\) mutant molecules not only bind to β2m during biosynthesis with similar efficiencies to K\(^b\)WT molecules, but that they stay associated with β2m for similar periods of time once at the cell surface.
Figure 3-9: Co-immunoprecipitation of H-2K$^b$ and $\beta_2$-microglobulin.

L cell fibroblast pulse-chases showing $\beta_2$m co-immunoprecipitating with H-2K$^b$. Densitometry measurements of this and other autoradiographs showed that the density ratios between the K$^b$ and $\beta_2$m bands were similar throughout the course of the experiments and were also similar between wild-type K$^b$, K$^k$, and mutant K$^b$ molecules.
3.4 Discussion

The results presented in this chapter demonstrate subtle but consistent phenotypic differences between wild-type K\textsuperscript{b} and cytoplasmic mutant K\textsuperscript{b} molecules expressed in L cell fibroblasts. The fact that mutant K\textsuperscript{b} molecules were expressed at higher overall cell surface levels compared to K\textsuperscript{b}WT molecules is consistent with a lack of internalization, as has been previously reported in T cells for class I molecules containing exon VII deletions (188). Fibroblasts are known to internalize their class I molecules relatively slowly, making internalization studies technically difficult. However, crosslinking of surface class I molecules has been shown to increase this rate significantly (253). This mechanism of class I internalization, in contrast to T cell class I internalization, takes place via non-coated surface invaginations that are endocytosed and subsequently fuse with lysosomes. It is possible that cytoplasmic mutations could inhibit this process by interfering with the recognition of the class I intracellular region by internalization components. While internalization of the K\textsuperscript{b} mutants was not examined in L cell fibroblasts, it was examined in T cells (see Chapter 4).

Another difference observed between fibroblasts transfected with wild-type versus mutant K\textsuperscript{b} was in their effects on endogenous class I expression levels. L cell transfectants expressing the wild-type K\textsuperscript{b} gene showed reduced K\textsuperscript{k} and D\textsuperscript{k} surface levels that correlated well with increasing K\textsuperscript{b} surface levels. However, despite expressing significantly higher overall K\textsuperscript{b} cell surface levels, fibroblasts transfected with mutant K\textsuperscript{b} genes expressed endogenous class I alleles K\textsuperscript{k} and D\textsuperscript{k} just as well, if not better, than
untransfected L cells. This phenotype, although less obvious in the fibroblasts, was much more clearly defined in lymphocytes derived from transgenic mice expressing the same K\textsuperscript{b} mutants (see Chapter 4). Possible explanations for this phenomenon can be found in the Chapter 4 discussion (Section 4.4), as well as in Chapter 6.

Differences were also observed in the abilities of the K\textsuperscript{b}WT and mutant K\textsuperscript{b} molecules to bind exogenous peptides. In the case of the ovalbumin-derived K\textsuperscript{b}-binding peptide (OVA), this difference in binding was directly measured using an antibody specific for K\textsuperscript{b}-OVA complexes. These experiments demonstrated that in transfectants expressing comparable K\textsuperscript{b} surface levels, K\textsuperscript{b} mutant molecules bound 2 to 4 times more exogenous OVA peptide than K\textsuperscript{b}WT molecules. Similarly, incubation with the K\textsuperscript{b}-binding VSV N-peptide led to a larger increase in the number of K\textsuperscript{b} surface complexes in K\textsuperscript{b} mutant than in K\textsuperscript{b}WT transfectants, implying that more peptide was binding to K\textsuperscript{b} mutant molecules. These observations would be expected if a cytoplasmic tail-mediated mechanism existed to clear empty class I H chains from the cell surface following complex dissociation. Cytoplasmic mutations could conceivably result in a higher level of empty class I H chains building up at the cell surface, which would likely be more receptive to exogenous peptide binding. Most class I-specific monoclonal antibodies cannot detect empty H chains, largely due to the fact that most were raised against conformational-specific epitopes that can recognize only trimolecular (ie. \(\beta_2m\) and peptide-bound) complexes. Only antisera raised against cytoplasmic determinants can bind empty H chains, but these are not practical for examining cell surface expression using FACScan techniques.
An alternate explanation for the increased binding of OVA peptide to mutant K\(^b\) molecules takes into account the spectrum of peptides naturally bound to the K\(^b\) molecules at the surface of the L cell fibroblasts. If mutant K\(^b\) molecules bound to lower affinity peptides than K\(^b\)WT molecules during their biosynthesis, it is conceivable that the OVA peptide (which binds to K\(^b\) with a very high affinity) may more effectively compete for binding to K\(^b\) mutant molecules. Characterizing the spectrum of peptides naturally bound to K\(^b\)WT and K\(^b\) mutant molecules in these cells will be essential for answering this question.

Experiments involving \(\beta_2m\) detection and binding also revealed some interesting differences between wild-type and mutant K\(^b\) molecules. The first observation was that of an altered surface \(\beta_2m\) to total class I ratio in K\(^b\) mutant transfectants, as measured by FACScan. In untransfected and K\(^b\)WT-transfected L cells, this ratio was measured to be 0.72 ± 0.03 (n=3). It was consistently lower in all of the K\(^b\) mutant-expressing transfectants, averaging 0.53 ± 0.06 (n=3). Since conformationally-specific antibodies were used to measure class I surface expression, by definition they detected only class I H chains complexed to \(\beta_2m\) and peptide. Thus, the lower amount of \(\beta_2m\) observed at the mutant transfectants’ cell surface relative to total class I is puzzling. Other molecules can also bind \(\beta_2m\), but there is little reason to suspect that their expression or \(\beta_2m\) binding is differentially affected by the presence of wild-type or mutant K\(^b\) molecules.

One explanation that is consistent with the empty H chain clearance mechanism hypothesis is that the \(\beta_2m\)-specific antibody may have recognized murine but not bovine
β2m. Conformation-specific monoclonal antibodies, on the other hand, can recognize class I complexes whether murine or bovine β2m is bound (254). This would imply that mutant K<sup>b</sup> molecules are bound to significantly higher amounts of bovine β2m than are K<sup>b</sup>WT molecules. It is possible that exogenous bovine β2m serves to stabilize mutant K<sup>b</sup> molecules that have undergone dissociation from murine β2m, yet cannot be internalized normally as a result. Consistent with this notion, fetal bovine serum deprivation led to a larger decrease in conformational surface K<sup>b</sup> mutant complexes than in K<sup>b</sup>WT complexes (particularly in the ΔVII, ΔSTSS, and ΔYSTSS mutants). Since densitometry experiments showed that K<sup>b</sup> mutant and K<sup>b</sup>WT molecules associated with similar efficiencies to murine β2m during biosynthesis and subsequent expression at the cell surface, any bovine β2m association would have to occur subsequent to murine β2m dissociation.

Pulse-chase analyses of the L cell transfectants failed to detect any significant differences in the maturation rates or half-lives of K<sup>b</sup>WT and mutant K<sup>b</sup> complexes. The maturation result is not surprising since entire cytoplasmic tail deletions have been demonstrated to have minimal effects on class I maturation rates (214). Since conformational-dependent antibodies were used in these experiments, the similar half-lives indicate that dissociation rates for the complexes were similar if not identical between mutant and wild-type K<sup>b</sup> molecules. What it doesn’t reveal is what happens to the K<sup>b</sup> H chains after complex dissociation. If cytoplasmic mutations prevent optimal recognition, internalization, and degradation of empty class I H chains, it is possible that these molecules stay at the cell surface for an unusually extended period of time. Repeating these pulse-chase
experiments using a cytoplasmic domain-specific antiserum is more likely to reveal whether there is a significant difference in KWT and Km mutant H chain half-lives.

Comparison of KWT- and Km mutant-expressing fibroblast clones revealed that equal Kb cell surface expression does not correlate with equal Kb synthesis rates. In pulse-labelling experiments, mutant clones consistently synthesized less Kb during the pulse period. This observation again would be expected if cytoplasmic tail mutants were internalization-defective. Alternatively, it could indicate that KWT-expressing clones molecules maintain a larger intracellular pool of Kb than do Km mutant-expressing clones, which presumably express most of their Kb at the cell surface. Either way, it is clear that the dynamics of class I expression in fibroblasts were altered significantly by cytoplasmic mutations.

Up to this point, the discussion has not distinguished a great deal between the four different Kb cytoplasmic mutants. This is mainly due to the fact that most of the mutants displayed similar phenotypes in all of the experiments presented. The AY mutants generally showed the mildest phenotypic differences compared to wild-type Kb, and the exon VII mutants (AVII, ASTSS, and AYSTSS) generally showed the largest differences. This supports previous studies that have implicated Exon VII determinants as being essential for cell surface internalization. It also implicates phosphorylation as having a possible role in any internalization process, since the ASTSS mutant contains point mutations to residues known to constitute class I phosphorylation sites.
The apparent molecular weight (MW) differences observed when \[^{35}\text{S}\]-labeled immunoprecipitates of K\(^{b}\)WT and K\(^{b}\) mutant molecules were run on SDS-PAGE were somewhat unexpected. The K\(^{b}\)WT, ΔY, and ΔVII mutants all ran at the expected MW (based on the mutations made), but the ΔSTSS and ΔYSTSS mutants ran at MWs slightly higher and slightly lower than the ΔVII molecules, respectively. This is somewhat puzzling, because the ΔVII molecules are predicted to contain a deletion of thirteen amino acids, whereas the ΔSTSS and ΔYSTSS molecules are predicted to be full-length H chains containing only point mutations. This may be indicative of one of several possibilities: since exon splicing is known to occur in class I mRNA, the exon VII point mutations may have created alternative splice junctions which led to spliced mRNA transcripts. However, if exon VII was being spliced out in the ΔSTSS and ΔYSTSS mutants, we would expect them to run at MWs identical to the ΔVII mutant, which is not the case. Furthermore, computer analyses of the mutant and wild-type DNA sequences using three different splice site prediction programs failed to predict any extra splice junctions due to the point mutations made.

One possibility is that a post-translational cytoplasmic modification is prevented by the point mutations. An obvious candidate for this modification is phosphorylation, but is probably unlikely due to the fact that normally only a small proportion of class I molecules is phosphorylated in cells at any given time (207). Other modifications to the class I cytoplasmic tail have been reported, including an acidic modification that has been mapped to exon VI (255). The authors of this study speculated that several candidate post-translational modifications such as asparagine deamination, N-acetylation or N-
methylation of arginine, ADP-ribosylation, or tyrosine sulfation may be responsible. Interestingly, computer motif searches have revealed that exon VII also contains a predicted myristylation site.

Exon VII-specific antisera appears unable to immunoprecipitate the ΔSTSS and ΔYSTSS mutants (data not shown). However, interpreting this result is difficult because any lack of exon VII recognition may have been due to the four amino acid substitutions rather than the deletion of the entire exon. Furthermore, this antisera reacts only with a subset of Class I molecules (those that are not bound to β2m) and it cross-reacts with K^k. Exon VIII-specific antisera, on the other hand, did recognize the ΔSTSS and ΔYSTSS mutant molecules, indicating that a gross truncation was not responsible for the MW differences observed. Similar MW aberrations were observed for ΔSTSS and ΔYSTSS molecules in lymphocytes derived from transgenic mice (see Chapter 4). The implications of these findings will be discussed further in Chapter 4 and Chapter 6.

In conclusion, the data presented in this chapter provides evidence that is consistent with, but does not prove, the existence of a mechanism that internalizes empty Class I molecules based on recognition of conserved cytoplasmic determinants. While a possible role for phosphorylation in mediating this process is implicated by the data, the lower-than-expected apparent MWs observed for the ΔSTSS and ΔYSTSS mutants will have to be further characterized before any definitive conclusions can be reached. It cannot be ruled out that the serine and threonine residues may simply be important for folding of the cytoplasmic domain, independent of phosphorylation.
The fact that $K^b$ mutants were better able to bind exogenous peptides compared to wild-type $K^b$ molecules may provide a clue to the evolutionary pressures that contributed to the high level of conservation of certain parts of the cytoplasmic domain. As discussed in the introduction to this chapter, it is likely not to the cell’s advantage to indiscriminately display peptides that are acquired from exogenous sources since it could lead to inappropriate targeting by CTL in vivo. It is difficult to say whether the physiological peptide concentrations found in vivo would be sufficient to cause inappropriate CTL killing. However, if this were the case it may manifest itself in some form of autoimmunity within an animal carrying a class I gene containing similar cytoplasmic mutations.

The next chapter describes the generation and analysis of transgenic mice expressing either the $K^b$WT or each of the four cytoplasmic $K^b$ mutants. Chapter 5 examines antigen presentation function of the $K^b$ mutants, both in the L cell fibroblast transfectants described in this chapter and in the transgenic mice, using the $K^b$-restricted vesicular stomatitis virus (VSV) system.
Chapter 4 – Generation and Analysis of K\textsuperscript{b} Transgenic Mice (Expression and Trafficking)

4.1 Introduction

Previous studies designed to elucidate the role of the MHC class I cytoplasmic domain have largely been conducted using transfected cell lines. However, this approach has proved limited in terms of providing answers regarding the cytoplasmic tail's physiological significance. Since MHC class I molecules are so intimately involved with immune system function, particularly in CD8\textsuperscript{+} T cell selection and activation, it was of interest to us to create an \textit{in vivo} system to study the cytoplasmic domain. It was hoped that by examining MHC class I cytoplasmic mutants within a whole organism and in the context of a complete immune system, the physiological significance of the cytoplasmic region could be clarified.

To this end, we generated transgenic mice expressing wild-type or cytoplasmic tail mutants of the H-2 K\textsuperscript{b} molecule. The goal of these studies was to understand the \textit{in vivo} relevance of the highly conserved class I cytoplasmic tyrosine and serine residues, as well as to elucidate the functional relevance of the naturally-occurring Exon VII splicing variant. This chapter covers the generation and breeding of the transgenic mouse lines and also describes experiments designed to study the expression, trafficking and internalization of MHC class I in transgenic murine lymphocytes. Chapter 5 examines the
abilities of the same transgenic mice to generate functional CTL responses to a K<sup>b</sup>-restricted viral epitope.

The work in this chapter can be divided into three broad sections. The first comprises a detailed study of splenocyte and peripheral blood leukocyte (PBL) class I surface expression in all of the transgenic mouse lines. The study followed MHC class I expression across several generations of breeding and includes data on all founder mice, hemizygous offspring, and selected mice bred to homozygosity. These experiments revealed a fundamental role for the cytoplasmic tail in regulating normal MHC class I cell surface expression in murine splenocytes and peripheral blood leukocytes. The second part of this chapter describes experiments utilizing metabolic $^{35}$S labeling to characterize the K<sup>b</sup> mutants in terms of their size, maturation rates, and half-lives. Comparisons were made between class I synthesis rates in wild-type and mutant-expressing splenocytes and correlations were made with surface expression levels.

The third section examines constitutive class I internalization in activated T cells. Since it had been previously shown in T lymphoblastoid cell lines that MHC class I internalization is dependent on exon VII determinants, we were interested in whether these determinants could be narrowed down to the conserved amino acids targeted in our K<sup>b</sup> point mutants. These experiments, in addition to confirming the earlier studies, also provided the first evidence that a tyrosine-based endocytic sorting signal present in the cytoplasmic domain of class I molecules is required for their constitutive internalization in activated T cells.
4.2 Materials and Methods

4.2.1 Transgenic Mice

The DNA constructs described in Chapter 2 were purified on cesium chloride gradients and the wild-type and mutant H-2K\(^b\) genes were subsequently excised from their original cloning vector, pBR322, by an Eco RI digestion followed by agarose gel purification. The DNA was diluted to 1 \(\mu\)g/mL and microinjected into fertilized murine oocytes (derived from a C3H/C57 mating) before being transplanted into the uteri of pseudo-pregnant female mice. Transgenic offspring were originally selected by Southern blot analysis showing increased levels of the K\(^b\) gene in the mouse tail DNA (Figure 4-1). Jean-Pierre Julien at McGill University in Montreal produced the transgenic founder mice and performed the screening by Southern blot. This approach provided ten founder mice containing the K\(^b\) wild-type transgene, ten founders containing the \(\Delta\)VII construct, nine founders containing construct \(\Delta\)STSS, four founders containing the \(\Delta\)Y construct, and one founder mouse containing the K\(^b\) mutant \(\Delta\)YSTSS. These founder mice (all of H-2\(^{b\kappa}\) haplotype) were tested for their ability to express the K\(^b\) transgene at increased protein levels by FACScan analysis of peripheral blood leukocytes and it was found that approximately 80% of the mice could. Three founder mice from each group covering a range of transgene expression levels were bred to C3H mates. F1 offspring were tested for their MHC haplotypes and mice expressing the K\(^b\) transgene on an H-2\(^\kappa\) background were chosen for future breeding. These mice were identified by the absence of D\(^b\) on the surface of their PBLs in addition to showing an increased level of K\(^k\) by FACScan.
Figure 4-1: Southern blot screen for transgenic mice.

Tail DNA derived from offspring of pseudo-pregnant females implanted with fertilized oocytes (previously microinjected with the K\textsuperscript{b}WT gene) was cut with Bgl I and probed with a \textsuperscript{32}P-labeled, K\textsuperscript{b}-specific probe. K\textsuperscript{b}WT (shown here), \Delta Y, \Delta STSS, and \DeltaYSTSS mouse DNA (not shown) was probed with labeled secondary PCR fragment \DeltaSTSS (see Table 2-2). Potential \Delta VII transgenics were probed with labeled secondary PCR fragment \Delta VII (data not shown). Arrows indicate transgenic animals containing extra copies of the K\textsuperscript{b} gene (compared to the majority of mice, who contain one copy as part of their C3H/C57 H-2\textsuperscript{b}k MHC background). Generation and screening of mice was performed by Jean-Pierre Julien and Gaétan Gagnon of McGill University, Montréal.
analysis (as is observed in H-2\textsuperscript{k} control mice compared to H-2\textsuperscript{bk} control mice, refer to Figure 4-3). All subsequent breedings were made between mice of the H-2\textsuperscript{k} background, either by back-crossing transgenic mice to C3H mice to produce hemizygous animals or by mating transgenic H-2\textsuperscript{k} siblings to produce doubly transgenic, homozygous animals (Figure 4-2). All mice used in the experiments described were individually tested for haplotype and MHC class I allelic expression levels by PBL FACScan analysis (see below).

4.2.2 Cells and Antibodies

Mice were sacrificed and splenocytes were obtained by homogenization of spleens in RPMI-1640 supplemented with 10% fetal calf serum (FCS). Splenocytes were spun down at 500 x g and resuspended briefly in 0.83% ammonium chloride to lyse red blood cells before re-equilibrating with RPMI/10% FCS and spinning down again. Two subsequent washes in cold PBS/2% FCS preceded FACS staining of splenocytes. Activated T cells were obtained by culturing splenocytes at 2x10\textsuperscript{6} cells per mL in RPMI-1640 supplemented with 10% FCS, 2 mM L-glutamine, 10 mM HEPES, and 2 µg/mL concanavalin A (Sigma). Peripheral blood leukocytes were obtained from halothane-anaesthetized mice by an intra-ocular bleed. 100 µl of blood was mixed with 75 µl 1M heparin in PBS, followed by 825 µl of PBS before being applied to a 2 mL Ficoll gradient and spun for 15 minutes at 500 x g. PBLs were recovered and washed twice in cold PBS/2% FCS before FACS staining with AF6.88.5.3 (256), 28.14.8S (257), and
16.3.1N (258), conformation-dependent mouse mAbs (of the IgG2a isotype) specific for extracellular domains of H-2 K\textsuperscript{b}, D\textsuperscript{b}, and K\textsuperscript{k}, respectively.
Transgenic founder mice, which expressed the K\(^b\) transgene on a C57 / C3H (H-2\(^{b/k}\)) background, were bred to C3H mates (depicted as white mice) in order to breed out the K\(^b\) gene that is normally part of the C57 genome. Offspring were screened by evaluating peripheral blood leukocyte (PBL) Class I surface expression. Mice that expressed K\(^b\) but not D\(^b\), and that showed an increased K\(^k\) expression compared to their parents or C3H/C57 controls, were chosen to establish the transgenic mouse lines. These hemizygous transgenic animals (on an H-2\(^k\) background) were backcrossed three more times to C3H mice before attempting to generate homozygous transgenic mice by hemizygous sibling matings. Homozygous animals were selected by an increased PBL K\(^b\) surface expression in addition to a decreased level of endogenous Class I surface expression, compared to the hemizygous parents. Hemizygous transgenic mice are shown as light grey, homozygous mice are depicted as dark grey.
Selected Tg founder mice

C3H mice

H-2^{b/k} K^{b}Tg^{+/--}

25%

H-2^{k/k} K^{b}Tg^{+/-}

25%

Hemizygous transgenics

*[Backcross to C3H mice for three additional generations before mating to establish homozygous transgenics]*

Hemizygous Tg

Sibling Mating

K^{b}Tg^{+/--}

25%

K^{b}Tg^{+/-} (hemizygous Tg)

50%

K^{b}Tg^{+/+} (homozygous Tg)

25%

K^{b}Tg^{--} (non-transgenic)
4.2.3 FACS Staining

Peripheral blood leukocytes and splenocytes were analyzed by flow cytometry analysis using either AF6.88.5.3, 28.14.8s, or 16.3.1N as primary antibodies. Cells (1-2 x 10^5) were incubated with antibody for 30 min at 4°C, washed, then incubated with biotin-conjugated goat anti-mouse IgG_2a for 30 min (Southern Biotechnology), washed, and finally incubated with streptavidin-fluorescein isothiocyanate (Jackson ImmunoResearch Laboratories) for 30 min. Cells were then washed, fixed and analyzed by FACScan (Becton-Dickinson). Forward and side scatters were obtained and cells were gated to exclude the majority of red blood cells and to include lymphocyte populations. Gated cells were analyzed at 5 x 10^3 cells per histogram. Double-labeling experiments utilized H-2 K^b- and K^k-specific monoclonal antibodies directly conjugated to FITC (Cedarlane), a mouse CD4-specific mAb conjugated to phycoerythrin (PE), a mouse CD8-specific mAb conjugated to biotin, a mouse B220-specific mAb conjugated to biotin (Pharmingen), and finally, streptavidin-PE to detect bound primary antibody (Cedarlane). Compensation was performed on the FACScan analyzer FITC and PE channel gains before obtaining scatter plots of lymphocyte-gated cells.

4.2.4 Splenocyte Pulse-chases

Splenocytes were starved in methionine and cysteine-free media for 20 min, pulsed with [^{35}S]methionine at 300 μCi/mL for 20 min, and chased in medium containing excess methionine (0.5 mM) and cysteine (0.5 mM). At the indicated time points, 1 x 10^7 splenocytes were collected, washed in cold phosphate-buffered saline, and lysed in 1%
Nonidet P-40 (NP-40) buffer (120 mM NaCl, 4 mM MgCl₂, 20 mM Tris-HCl pH 7.6) containing 40 µg/mL PMSF for 20 min at 4°C. After cell nuclei had been removed by centrifuging for 15 minutes, cell lysates were TCA-precipitated and equal counts were precleared with normal rabbit serum and Protein A-sepharose (Pharmacia) prior to immunoprecipitation with mAbs AF6.88.5.3 or 16.3.1N. Thereafter, Protein A-sepharose beads were added to bind immune complexes. The beads were washed four times and at this stage immunoprecipitated proteins (depending on the experiment) were subjected to endoglycosidase treatments. Samples were boiled in 30 µl of 3.5 % SDS / 30% sucrose / 80 mM Tris (pH 8.8) / 0.01% bromophenol blue solution containing 20 mM dithiothreitol for 5 min before adding iodoacetamide to 200 mM and separating proteins on a 10-15% gradient gel by SDS polyacrylamide gel electrophoresis (PAGE). The gels were fixed, enhanced, dried and exposed to autoradiographic film.

4.2.5 Endocytosis Experiments

Splenocytes were harvested, depleted of RBC by resuspending in 0.83% ammonium chloride, depleted of B cells using CD19-specific magnetic beads (Miltenyi Biotec), and cultured for 3 days in RPMI supplemented with 10% FCS and 2 µg/mL concanavalin A. Activated T cells were then washed and exposed to either K⁺- or K⁻-specific monoclonal antibodies conjugated either to FITC (Cedarlane) or ¹²⁵I (lactoperoxidase method) for 5 min at room temperature. Cells were washed and warmed to 37°C and aliquots were taken at the time points indicated. Control cells underwent the same treatments but were kept at 4°C during the course of the experiment. Cells exposed to fluorochrome-labelled
antibodies were immediately fixed in 1.5% paraformaldehyde and examined under a confocal fluorescent microscope. Cells were photographed by taking a Z-series of images which were later selected and compiled to show the total intracellular fluorescent vesicle content. Cells treated with $^{125}$I-conjugated antibodies were split into two aliquots, one of which was resuspended in normal RPMI medium and one which was resuspended in RPMI buffered to pH 2 with HCl. Cells were washed and spun down and cell pellets were counted in a gamma counter to quantitate internalized $^{125}$I. The percentage of internalized radioactivity was calculated in the following manner:

Percent internalized = experimental – minimum / maximum – minimum

where minimum count measurements were obtained by treatment at 4°C with acid stripping, maximum counts were obtained following 37°C with no acid treatment, and experimental counts were obtained following 37°C with acid treatment.

4.3 Results

4.3.1 PBL Class I Expression in Transgenic Founder Mice

4.3.1.1 Transgenic K$^b$ Expression

FACScan analysis of peripheral blood leukocytes (PBLs) derived from the transgenic founder mice immediately revealed a difference in K$^b$ cell surface expression levels between the K$^b$WT mice and the ΔVII, ΔSTSS, and ΔY mutant-expressing mice.
Whereas the $K^b$WT founder mice measured an average fluorescence of $534 \pm 66$ arbitrary fluorescence units (AFU), the $\Delta$VII mice measured an average of $757 \pm 159$ AFU, the $\Delta$STSS mice averaged $810 \pm 156$ AFU and the $\Delta$Y mice averaged $868 \pm 65$ AFU. Thus, the $\Delta$VII, $\Delta$STSS, and $\Delta$Y mice expressed $K^b$ molecules on the PBL cell surface at an average of 42%, 52%, and 63% higher levels, respectively, compared with $K^b$WT mice. The lone $\Delta$YSTSS mutant measured a PBL surface $K^b$ level of 582 AFU (Figure 4-4). Similar results were observed for splenocytes from the founder mice (data not shown).

4.3.1.2 Endogenous Class I Expression

In order to establish a total MHC class I surface expression profile of the transgenic founder mice, PBL expression of endogenous class I alleles $K^k$ and $D^b$ was also examined by FACScan. All of the founder mice contained one copy of each of the $K^k$, $D^k$, and $D^b$ genes in addition to varying levels of $K^b$ transgene. By comparing the class I expression in transgenic founder mice and C3H/C57 control mice (containing the same genetic background with no $K^b$ transgenes), another significant phenotypic difference became apparent. While most of the $K^b$WT founder mice showed a significant reduction in the PBL surface expression of both $D^b$ and $K^k$, the majority of the $K^b$ mutant founder mice showed only a small reduction in these endogenous alleles at comparable levels of $K^b$ transgene surface expression (see Figure 4-3 and 4-4). Plotting the endogenous class I cell surface expression levels against the transgenic $K^b$ surface expression levels for the founder mice revealed not only that cell surface expression of transgene-encoded $K^b$ was
Figure 4-3: Class I surface expression in PBLs of transgenic founder mice.

FACScan profiles showing peripheral blood leukocyte (PBL) cell surface levels of MHC Class I alleles $K^b$, $D^b$, and $K^k$ in C57, C3H, and C57 x C3H F1 control mice (top three rows) and selected $K^bWT$, $\Delta$STSS and $\Delta$VII transgenic founder mice (bottom three rows). Numbers indicate mean surface fluorescence as measured in arbitrary fluorescence units (AFU).
Figure 4-4: PBL MHC Class I surface expression in transgenic founder mice.

For each founder mouse (H-2b/k background), transgene-encoded K^b surface expression is plotted against expression of endogenously-encoded D^b. All mice shown contained one copy of the D^b gene and variable numbers of copies of the K^b transgenes. The expression levels of all three endogenously-encoded Class I alleles (including K^k and D^k) were similarly affected by the presence of the K^b transgenes (data not shown). Founder mice within the dotted circle had Class I expression profiles indistinguishable from C57xC3H F_1 controls, implying that their transgenes were not expressed. The data shows one experiment that is representative of three replicate experiments. All data is in mean arbitrary fluorescence units (AFU).
inversely proportional to the cell surface levels of endogenous alleles D\textsuperscript{b} and K\textsuperscript{k} in both the K\textsuperscript{b}WT and mutant founder mice, but that there was a striking difference between the K\textsuperscript{b}WT and mutant transgenic mouse groups in the level of K\textsuperscript{b} cell surface expression at which this reduction in D\textsuperscript{b} and K\textsuperscript{k} was seen to occur. The K\textsuperscript{b} cell surface expression levels at which endogenous class I surface levels were reduced was significantly higher in all of the cytoplasmic tail mutants, compared to K\textsuperscript{b}WT mice.

4.3.1.3 Selection of founder mice for breeding

The transgenic MHC founder profiles also revealed that each mouse group contained a range of transgene expression levels. These included high, medium, low, and partial K\textsuperscript{b} expressors. Partial expressors demonstrated a mosaic-like pattern of transgene expression whereby only a subset of PBLs expressed the K\textsuperscript{b} transgene. Curiously, this partial transgene expression phenomenon occurred predominantly amongst the ΔSTSS and ΔVII founder mice. Most commonly, one-third of the PBLs from these mice were K\textsuperscript{b}-negative but this percentage varied widely. The phenomenon was also observed to occur (albeit to a lesser extent) amongst the K\textsuperscript{b}WT-expressing founder mice. This mosaic expression phenomenon has been reported elsewhere for other class I transgenic mice (259) but has never been adequately explained. For the purposes of this thesis work, it was decided early in the project not to breed and study the mice that demonstrated partial expression of the K\textsuperscript{b} transgene. This decision was based primarily on the future interpretation of experimental results, which could potentially be more difficult and confusing when having to take mosaic transgene expression into account.
Therefore, the first criteria to choose which of the founder mice to keep and breed for establishing the transgenic mouse lines was a PBL transgene expression of 90% or higher. For each of the groups K\(^b\)WT, AVII, ASTSS, and AY, three founder mice were selected to backcross to C3H mice. They were selected to represent low, medium, and high transgene expressors, based on the observed K\(^b\) and endogenous class I PBL cell surface levels. All of the transgenic lines, including the lone AYSTSS founder, were bred successfully onto an H-2\(^k\) background.

4.3.2 PBL Class I Expression in Transgenic Offspring

Crossing the selected transgenic founder mice with C3H mice generated hemizygous animals that expressed the K\(^b\) transgene on an H-2\(^k\) MHC background. This step was important in order to 'breed out' the endogenous H-2\(^b\) genes (one of which was the K\(^b\) wild-type gene) from the founder lines. The resulting hemizygous H-2\(^k\) transgenics contained two copies each of the K\(^k\) and D\(^k\) genes, in addition to varying amounts of K\(^b\) transgene. PBL surface expression of these three class I alleles was measured by FACScan for all of the offspring generated from the transgenic matings. The results are summarized in Figure 4-5.
Figure 4-5: PBL MHC Class I expression in hemizygous transgenic mice.

Surface expression of MHC Class I in PBLs from hemizygous transgenic mice bred onto an H-2\(^k\) background. All mice shown have two copies each of the K\(^k\) and D\(^k\) genes in addition to variable numbers of integrated K\(^b\) transgenes. Similar results were obtained when K\(^b\) surface expression was plotted against D\(^k\) (data not shown). Splenocytes isolated from the same mice provided nearly identical results (data not shown). The data shows one experiment that is representative of at least five replicate experiments. Axis values are in mean arbitrary fluorescence units (AFU).
The MHC class I profiles of these mice confirmed the trend described for the founder mice, demonstrating that the same phenotypic differences could be observed in the absence of an endogenous K\(^b\) gene. PBLs and splenocytes from K\(^b\)WT-expressing mice demonstrated a sharp dropoff in endogenous class I expression (K\(^k\) and D\(^k\)) in response to increasing transgene levels and seemed to reach a upper limit of K\(^b\) surface expression (~550 AFU) which none of the K\(^b\)WT-expressing animals surpassed. By contrast, surface K\(^b\) in the cytoplasmic mutant mice reached significantly higher levels and mutant transgene expression seemed to have a less drastic effect on endogenous class I expression (Figure 4-5).

In order to simplify breeding, sibling matings of mouse lines were done to produce mice homozygous for transgene expression and thus containing twice as many K\(^b\) transgene copies as the parents. These mice provided an opportunity to further characterize the phenomenon of endogenous class I down-regulation in response to class I transgene expression. PBL surface class I analysis of parents and offspring produced some surprising results and provided further insight into the dynamics of class I expression. The results are depicted in Figure 4-6.

Two K\(^b\)WT mouse strains bred to homozygosity demonstrated relatively small increases in PBL K\(^b\) surface levels compared to their hemizygous parents while K\(^k\) levels decreased significantly. Most of the mutant K\(^b\)-expressing transgenic mice, however, showed a much different expression trend when bred to homozygosity (Figure 4-5).
Figure 4-6: PBL MHC Class I expression in homozygous transgenic mice.

Comparison of PBL MHC Class I surface expression in hemizygous and homozygous transgenic mice. Smaller symbols represent hemizygous animals and larger symbols their homozygous offspring. Arrows connect parents and offspring. All mice have been bred onto an H-2^k MHC background. Similar results were obtained when transgenic K^b surface expression was plotted against D^k (data not shown). The data represents one of at least three replicate experiments for each strain. Axis values are in mean arbitrary fluorescence units (AFU).
The ΔSTSS, ΔYSTSS, and ΔVII mice demonstrated PBL surface K\(^b\) levels which were nearly double that of their hemizygous parents while only showing a minor decrease in K\(^k\) expression. A ΔY-expressing transgenic line showed a more moderate 35% increase in surface K\(^b\) in response to transgene doubling while also decreasing K\(^k\) levels by almost 50%. One hemizygous ΔVII mouse that showed a very high transgene expression was also bred to homozygosity, but class I expression was seen to change very little other than a further small decrease in an already low level of K\(^k\) surface expression (Figure 4-6).

### 4.3.3 Characterization of Transgenic Effects on Endogenous Class I Expression

#### 4.3.3.1 Heterogeneous Down-Regulation in K\(^k\) Surface Expression

FACScan analysis of transgenic PBLs and splenocytes revealed another feature of MHC class I expression in these transgenic mice. The decrease in endogenous K\(^k\) surface expression caused by increasing amounts of K\(^b\) transgene did not occur in a homogeneous fashion. As shown in Figure 4-7A, mice with little or no transgene expression showed a characteristically narrow histogram peak for K\(^k\), representing a relatively homogeneous cell population with respect to K\(^k\) expression. As the level of transgene expression increased (as judged by K\(^b\) surface expression), the PBLs and splenocytes began to separate into subgroups. At lower transgene expression levels, there appeared a small subpopulation (25%) of cells that was modestly reduced in K\(^k\) expression. At higher levels of K\(^b\) expression, the cells separated into a wide histogram
peak demonstrating a range of $K^k$ expression levels roughly evenly distributed from high to low. At still higher $K^b$ levels, the cells resolved into a distinct double peak with approximately 15-20% of the cells still expressing relatively high levels of $K^k$ while the remainder were at least tenfold reduced in $K^k$ surface expression. At the very highest levels of $K^b$ transgene surface expression, the entire population of PBLs and splenocytes was homogeneously low for surface $K^k$. This phenomenon of heterogeneous $K^k$ down-regulation was observed for some of the $K^b$ WT, ΔVII, and ΔY transgenic mice, but not for the ΔSTSS or ΔYSTSS mice. The main differences between the wild-type and mutant transgenics were the levels of $K^b$ surface expression at which the reduction in $K^k$ was seen to occur.

4.3.3.2 Novel T cell Subset with Increased Capacity for Class I Expression

To further characterize this heterogeneous reduction in endogenous MHC class I expression, a double-staining approach was used in order to identify the PBL cell subtypes and to correlate them with $K^k$ surface expression. We were especially interested in identifying the 15-20% of PBLs that were able to maintain a high level of endogenous class I expression despite simultaneously expressing relatively high amounts of $K^b$ transgene. Thus, PBLs from these mice were stained with $K^k$-specific antibody conjugated to phycoerethrin (PE) in addition to being stained with either CD4-, CD8-, or B220-specific antibody conjugated to fluorocein isothiocyanate (FITC) and subsequently analyzed by FACScan. The results (shown in Figure 4-7B) identify the PBL subset demonstrating an increased capacity for class I surface expression to be a mixture of
CD4+ and CD8+ T cells. Of the 42% of PBLs staining positive for CD4, approximately one quarter (11% of total PBL) showed high Kk surface levels. Likewise, of the 20% of PBLs staining positive for CD8, approximately one third (7% of total PBL) were Kk(hi). The remaining T cells were greatly reduced (~10-fold) in Kk expression. Of the B220+ PBLs (24% of total), virtually all were Kk(lo). Similar double-staining experiments on mice expressing lower transgene levels (Figure 4-7A, 2nd histogram) confirmed that B cells are the most susceptible subset of PBLs to show a reduction in endogenous surface class I due to increasing transgene expression (data not shown).
Figure 4-7: Characterization of K\textsuperscript{k} downregulation by K\textsuperscript{b} transgene expression.

(next page) (A) Heterogeneous downregulation of endogenous K\textsuperscript{k} expression in response to increasing K\textsuperscript{b} transgene expression. Numbers indicate surface K\textsuperscript{b} levels in PBLs from K\textsuperscript{b}WT and ΔVII transgenic mice (in mean AFU). Histogram peaks show resulting PBL K\textsuperscript{k} profiles. Arrow indicates subset of PBLs (15-20%) that are resistant to K\textsuperscript{k} downregulation. (B) Double staining of PBLs to identify subset of cells demonstrating increased capacity for MHC class I surface expression. K\textsuperscript{k} immunofluorescence was detected with FITC (FL1) and CD4, CD8, or B220 were detected with PE (FL2). NFA, no first antibody.
Figure 4-7A

Figure 4-7B
4.3.4 Molecular Characterization of K\textsuperscript{b} Molecules

In order to better characterize the cytoplasmic K\textsuperscript{b} mutants at the molecular level, \[^{35}\text{S}\] labelling and immunoprecipitations were performed on splenocytes derived from the transgenic mice. Pulse-chase experiments were done to examine three properties of splenocyte K\textsuperscript{b} molecules: first, their apparent size when run on SDS-PAGE; secondly, their maturation rates, as indicated by the increase in apparent MW as they acquire carbohydrate modifications and Endo H resistance in the cis-medial Golgi en route to the cell surface; third, their conformational half-lives in the cell, as judged by the rate of disappearance of the radioactive gel bands over the course of the experiment. Figure 4-8A shows the results of pulse-chase experiments done on splenocytes from several mouse lines.

The apparent MW of the K\textsuperscript{b} molecules when visualized on SDS-PAGE provided the one of the more surprising findings of these experiments. As expected, the mature K\textsuperscript{b}WT and ΔY molecules ran at very similar if not identical molecular weights (~ 45 kD) and the ΔVII mutant, 13 amino acids shorter, ran slightly faster at ~ 43 kD. However, the ΔSTSS and ΔYSTSS molecules appeared to show two isoforms. For both mutants, the less predominant isoform ran at the expected MW of ~ 44.5 kD, but the majority ran at a significantly lower MW of ~ 42.5 kD.
Figure 4-8A: Pulse-chases of transgenic and control splenocytes.

Splenocytes from indicated mouse strains were pulse-labeled with $[^{35}\text{S}]$ cysteine/methionine for 30 minutes and chased for various times before immunoprecipitation with the $K^b$-specific monoclonal antibody AF6.88.5. IPs were run on SDS-PAGE and exposed to autoradiographic film. *Lane 1*, 0 hours chase; *Lane 2*, 1 hour chase; *Lane 3*, 2 hours chase; *Lane 4*, 4 hours chase.
Figure 4-8B: Endoglycosidase treatments of K\textsuperscript{b} immunoprecipitates.

Splenocytes from K\textsuperscript{b}WT, ΔSTSS, and ΔYSTSS transgenic mice were pulse-labeled with [\textsuperscript{35}S]-cysteine/methionine for 30 minutes and chased for 0 or 2 hours before immunoprecipitation with the K\textsuperscript{b}-specific monoclonal antibody AF6.88.5. IPs were run on SDS-PAGE following either mock treatment (NT), Endoglycosidase H digestion (Endo H), or N-glycanase F digestion. Proteins were visualized by autoradiography. Lanes 1 & 2, K\textsuperscript{b}WT mouse (0 and 2 hours chase); lanes 3 & 4, ΔSTSS mouse (0h and 2h chase); lanes 5 & 6, ΔYSTSS mouse (0h and 2h chase).
To explore whether the lower MW isoforms in these mutants could be explained by
differential glycosylation, immunoprecipitated $K^b$ was digested with endoglycosidases
Endo H, Endo F, and N-glycanase. The results, shown in Figure 4-8B, indicated that the
apparent MW differences could not be attributed to glycosylation differences. Treatment
of $\Delta$STSS and $\Delta$YSTSS molecules with N-glycanase, which removes all glycosylation
modifications, results in the same two isoforms both running at a lower apparent MW.
$K^b$WT molecules, as expected, resolve to a single core protein upon treatment with N-
glycanase.

Maturation rates of Class I molecules can be monitored by their acquisition of
carbohydrate modifications which cause a shift to a higher apparent molecular weight as
seen on an SDS-PAGE gel. This form is resistant to treatment with Endo H, and
indicates that the Class I molecule has traversed the cis/medial Golgi compartment from
where it rapidly is transported to the cell surface. Analyses of the $K^b$ pulse-chases
showed that maturation rates appeared to be independent of cytoplasmic determinants but
were somewhat dependent on transgene expression level (Figure 4-8A). As might be
expected, mice expressing higher levels of $K^b$ transgene showed the slowest $K^b$
maturation rates.

The pulse-chase experiments were also used to analyze the conformational half-life of the
$K^b$ complex by measuring the disappearance of the autoradiograph bands using
densitometry. As in the fibroblast transfectants, the results failed to show a significant
difference in the conformational half-lives of $K^b$WT and cytoplasmic mutant $K^b$. 

148
molecules. The average half-life for wild-type and mutant K\(^b\) complexes in splenocytes was measured to be \(~6.5\) hours (densitometry results not shown).

### 4.3.5 Maturation Rates of Endogenous Class I

In order to better understand the differential effects on endogenous class I surface expression observed between the K\(^b\)WT and K\(^b\) mutant lines, \([^{35}S]\) pulse-chases were performed on splenocytes harvested from the transgenic mice. Equal numbers of splenocytes were pulse-labeled for 30 minutes followed by chases of 0 and 2 hours. The cells were then lysed and equal numbers of TCA counts were immunoprecipitated with K\(^b\)- or K\(^k\)-specific monoclonal antibodies followed by SDS-PAGE and autoradiography. The results of one representative experiment are shown in Figure 4-9.

In C3H control mice that express no K\(^b\), Endo H-resistant K\(^k\) comprises approximately 50% of the total labeled pool at the end of the pulse (0 hours) and 95% after a two hour chase. By comparison, two transgenic mice on the same MHC background, one expressing the wild-type K\(^b\) transgene and the other expressing the \(\Delta YSTSS\) mutant K\(^b\) transgene, showed quite different effects on the maturation of endogenous K\(^k\). Despite both expressing approximately equal amounts of K\(^k\) on the cell surface, the K\(^b\)WT transgenic mouse showed a much more substantial delay in the K\(^k\) maturation rate. By two hours only 45% of total labeled K\(^k\) had acquired Endo H resistance, compared to 85% in the \(\Delta YSTSS\) mutant mouse. The results were even more striking when it was considered that the \(\Delta YSTSS\) mutant mouse expressed over double the amount of K\(^b\) on
the splenocyte cell surface as the $K^b_{WT}$ mouse (as measured by FACScan) and produced approximately 25% more $K^b$ during the pulse period (as measured by spot densitometry). Similar results were observed for the $\Delta$VII and $\Delta$STSS $K^b$ mutants (data not shown). In addition to demonstrating slower maturation, immunoprecipitations of $K^b_{WT}$ splenocytes also showed a high molecular weight band (>200 kD) co-immunoprecipitating with $K^k$ as well as three or four other lower molecular weight bands that were especially prevalent at the 2 hour time point (data not shown). These bands did not appear in any of the immunoprecipitations from the mutant transgenic splenocytes. The lower molecular weight bands could possibly be breakdown products from $K^k$ degradation in the ER, or could represent stabilized interactions with other proteins caused by the overexpression of wild-type $K^b$ and resulting in slower transport through the secretory pathway. The high molecular weight bands co-immunoprecipitated with $K^k$ could also be explained using the latter reasoning. The absence of these bands in any of the $K^b$ mutant splenocytes in addition to the nearly normal $K^k$ maturation rate in these cells suggests that $K^b$ mutant molecules may not be traversing the same pathway to the cell surface as are $K^b_{WT}$ and $K^k$ molecules.
Figure 4-9: Comparison of K\textsuperscript{k} maturation in transgenic splenocytes.

(next page) Maturation of K\textsuperscript{k} molecules is delayed significantly by expression of the K\textsuperscript{b} wild-type transgene, but not the ΔYSTSS mutant transgene. [\textsuperscript{35}S] pulse-chases were performed on splenocytes, cells were counted, lysed and immunoprecipitated with K\textsuperscript{b}-specific (top panel) or K\textsuperscript{k}-specific monoclonal antibodies. Class I maturation can be monitored by the acquisition of carbohydrate moieties and conversion to a higher molecular weight form, indicating that the molecules have traversed the cis-medial Golgi compartment. Also indicated are splenocyte cell surface levels of K\textsuperscript{b} and K\textsuperscript{k} as measured by FACScan analysis (in AFU).
Figure 4-9

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FACScan results (AFU):

K^b: 43 kD
K^k: 43 kD
4.3.6 Internalization of MHC Class I Molecules in Activated T cells

In order to investigate whether the conserved cytoplasmic amino acids were responsible for internalization of class I molecules from the cell surface, a fluorochrome-antibody-based endocytosis study was initiated. Endocytosis of class I molecules via clathrin-coated pits in activated T cells is very rapid and is therefore an ideal system for monitoring their internalization. T cells derived from transgenic mice expressing comparable levels of surface K\textsuperscript{b} were enriched from spleens and cultured for three days in the presence of 2 \mu g/mL concanavalin A. The activated T cells were then stained with FITC-conjugated antibodies specific for either K\textsuperscript{b} or K\textsuperscript{k}, washed and incubated at 37\degree C for various time points before cooling the cells to 4\degree C and fixing in 1.5% paraformaldehyde. Observing the cells under a confocal fluorescence microscope revealed that K\textsuperscript{k} molecules were rapidly internalized in all transgenic mice and C3H control mice examined, as determined by the presence of punctate fluorescent vesicles inside the cells (see Figure 4-10A). K\textsuperscript{b} molecules, on the other hand, were only internalized to a significant extent in the K\textsuperscript{b}WT-expressing transgenic mice (Figure 4-10B) and C57 control mice. All of the K\textsuperscript{b} mutants examined, including the tyrosine point mutant, showed a severe reduction in the amount of intracellular fluorescence. The K\textsuperscript{b} mutants were observed to cap and form clusters but remained surface-bound.

Internalization rates were quantitated in a similar experiment using \textsuperscript{125}I-labeled antibodies against K\textsuperscript{b} and K\textsuperscript{k}. At time points following incubation of activated T cells with labeled antibody cells were washed in RPMI media buffered to pH 2.0 in order to strip remaining
surface-bound antibody and counted in a gamma counter to quantitate internalized radiation. The results, shown in Figure 4-11, confirmed the immunofluorescence observations.

Whereas K^k internalization rates were approximately the same in all of the activated T cells, K^b internalization rates were significantly reduced in the K^b mutants compared to K^bWT cells. Over the first 30 minutes of the experiment, C3H-derived T cells internalized K^k at a rate of 37% per hour. This was comparable to the K^k internalization rates measured for all of the transgenic T cells, which ranged from 31% to 42% per hour. In C57-derived T cells, K^b was internalized at a rate of 46% per hour. Activated T cells from the K^bWT transgenic mouse showed comparable K^b internalization rates of 51% per hour. By contrast, T cells derived from transgenic mice expressing cytoplasmic mutant K^b showed significantly reduced K^b internalization rates. AYSTSS T cells demonstrated the slowest K^b internalization rate (9% per hour), with ΔVII, ΔY, and ΔSTSS T cells measuring K^b internalization rates of 14%, 16%, and 19% per hour, respectively. Thus, K^b molecules with cytoplasmic alterations were internalized approximately three to five times slower that K^bWT molecules in activated T cells.
Figure 4-10: MHC Class I internalization in activated T cells.

(next two pages) Internalization of class I molecules in Con A-activated T lymphocytes derived from K^bWT, ΔY, and ΔSTSS transgenic mice. T cells were labeled with FITC-conjugated monoclonal antibodies specific for either (A) K^k or (B) K^b and allowed to internalize at 37°C for the indicated time points. Cells were photographed using confocal fluorescence microscopy to visualize internalized fluorescence. 600x magnification. Similar results were observed for the ΔVII and ΔYSTSS mutants (data not shown).
FIGURE 4-10A

$K^k$ internalization
FIGURE 4-10B

K^b internalization
Figure 4-11: Quantitation of Class I internalization in activated T cells.

Internalization of Class I molecules in Con A-activated T lymphocytes derived from transgenic and control mice. T cells were labeled with $^{125}$I-conjugated monoclonal antibodies specific for either (A) $K^b$ or (B) $K^k$ and allowed to internalize at 37°C for the time points indicated. T cells were then acid-stripped, washed, pelleted and counted in a gamma counter to quantitate internalized radioactivity. C57-derived T lymphocytes were used as control cells for $K^b$ internalization and C3H-derived T cells were used as controls for $K^k$ internalization. Error bars are standard error measurements for three replicate experiments.
4.4 Discussion

The results demonstrate that point mutation of the tyrosine residue alone was sufficient to severely impair the constitutive endocytosis of K\(^b\) molecules in concanavalin A-activated T cells, and was also sufficient to significantly affect class I surface expression levels in all transgenic splenocytes and peripheral blood leukocytes (PBLs) tested. All of the K\(^b\) mutants, but not wild type K\(^b\), were similarly impaired in their ability to undergo constitutive internalization in activated T cells. Wild-type K\(^k\), expressed endogenously by the same cells, was used as an internal control of class I internalization. Mice expressing the \(\Delta Y\) mutant also demonstrated splenocyte and PBL cell surface K\(^b\) levels consistently 60-70% higher than the maximal levels observed for K\(^b\)WT mice. The phenotype was similar in the \(\Delta STSS\) transgenic mice, but was even more dramatic in the some of the \(\Delta VII\)- and \(\Delta YSTSS\)-expressing mice, where the K\(^b\) cell surface levels were consistently more than double those of K\(^b\)WT mice.

Class I molecules in activated T cells are known to internalize rapidly via clathrin-coated pits (217). Our results suggest that, similarly to other membrane proteins, a tyrosine-based endocytic motif is responsible for their endocytosis. However, our results are also consistent with the idea that a phosphorylation event at an exon VII residue may control the recognition of the tyrosine motif by clathrin adaptin proteins. A similar mechanism has been demonstrated for the invariant chain (Ii), whereby point mutations in either the cytoplasmic di-leucine motif or a nearby phosphorylatable serine
residue abolished AP-1 adaptin binding and Ii-mediated trafficking of MHC Class II molecules to the endocytic compartment (42) (186). CD3 γ chains also contain a phosphoserine-dependent di-leucine motif that is important for downregulation of the T cell receptor (260).

It has been shown previously that deletion of exon VII abrogates endocytosis of class I molecules in a T lymphoblastoid cell line (188). The same study also demonstrated that deletion of exon VI (containing the conserved tyrosine residue) had no significant effect on internalization of class I. This observation, which contradicts our results, may be explained with the reasoning that such a gross deletion of eleven membrane proximal amino acids may cause the cell to target the molecule for rapid degradation. This could explain the apparently normal rate of internalization seen in exon VI deletion mutants. Deletions of exon VII, however, occur frequently as a natural splicing variation, and thus the cell would probably not recognize these molecules as abnormal.

Two recent reports described HLA-A and HLA-B molecules which, when point-mutated at the conserved cytoplasmic tyrosine residue, could no longer undergo HIV nef-induced MHC class I-induced downregulation (220) (221). Our results indicate that the tyrosine residue is not part of a cryptic sorting motif uncovered only by HIV- nef, but is a normal endocytic motif that is functional at a low level in resting lymphocytes but is upregulated upon activation of T cells. Since nef is known to be involved in modulation of T cell signal transduction pathways (261) (262), it is tempting to speculate that
increased phosphorylation of class I molecules by *nef* is the mechanism responsible for downregulation of surface expression.

It has long been known that class I molecules can be phosphorylated on at least one exon VII serine residue (187) and that naturally-occurring splicing variants delete this exon in several species thus far examined. This has led to speculation that deletion of exon VII may have implications for class I trafficking and function (211). To further explore this question, mutants of K\(^b\) were generated that contained complete deletions of exon VII. To address whether the conserved and phosphorylatable amino acid residues within exon VII were critical for its function, the serines were conservatively substituted by alanines and the threonine was replaced by isoleucine. This mutant should maintain the structural character of wild-type class I molecules, but should not be phosphorylatable. Our results suggest that an exon VII phosphorylation event may regulate recognition of the nearby exon VI-encoded tyrosine-based endocytic motif. This is supported by previous studies which demonstrated that phorbol esters increased class I phosphorylation in all cell types examined, but that phosphorylation only up-regulated class I internalization in lymphoid cells (208). It is conceivable that mutating four amino acids simultaneously could change the tail conformation sufficiently to abrogate recognition by internalization components independent of phosphorylation. However, since three of the amino acids (the serines) are extremely well-conserved we can at least conclude from our results that they are critical for exon VII function.
The fact that \( \text{K}^b \) expression in all splenocytes and peripheral blood leukocytes, and not just internalization in activated T cells, was affected by specific cytoplasmic mutations suggests that a fundamental mechanism of control of lymphocyte class I cell surface expression level had been altered. This is consistent with the high degree of evolutionary conservation demonstrated by the tyrosine and serine residues. Levels of class I on the surface of PBLs and splenocytes were observed to be remarkably consistent, not only within one mouse over its lifetime, but between mice of the same strain. We also noted that the amount of total class I molecules on the cell surface was approximately the same amongst all the strains examined. While expression levels of individual alleles varied, the total MHC class I usually fell within a consistently narrow window (Table 4-1). The same trend generally held true for the transgenic mice expressing wild-type \( \text{K}^b \), but a much different pattern was seen in the mice expressing mutated \( \text{K}^b \). Total lymphocyte MHC class I levels in these mice was consistently 60%-120% higher than that of control strains. In contrast, mice expressing the \( \text{K}^b \) wild-type transgene showed only a 15-25% increase in total cell surface MHC class I compared to \( \text{H-2}^b \) control strains. The mice with the highest total MHC class I expression were from the \( \Delta \text{VII} \) and \( \Delta \text{YSTSS} \) homozygous lines, with the \( \Delta \text{Y} \) and \( \Delta \text{STSS} \) lines demonstrating a slightly more modest phenotype.

Related to this phenomenon, the differential effects of mutant and wild-type transgene expression on the expression of endogenously-encoded \( \text{K}^k \) proved somewhat insightful in analyzing these transgenic mice. All of the transgenic mice examined contained two
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Table 4-1: Total MHC Class I expression in selected transgenic and control mice.

PBLs from each of the mice listed above were stained with FITC-conjugated monoclonal antibodies for surface expression of class I alleles $K^b$, $D^b$, $K^k$, and $D^k$ followed by quantitative analysis by FACScan. Mouse identifiers used are lo, med, and hi for low, medium, and high levels of $K^b$ transgene surface expression; alternatively, hemi and homo to indicate hemizygous mice and their homozygous offspring. All values are mean arbitrary fluorescence units (AFU). The estimated total class I expression was calculated as the sum of the AFUs from each of the individual class I alleles. Data is taken from a single experiment, but the trend shown was consistently observed in multiple experiments.
copies of the $K^k$ gene and varying amounts of $K^b$ transgene. Whereas wild-type $K^b$ caused a down-regulation of surface $K^k$ at relatively modest expression levels, mutant $K^b$ reached significantly higher expression levels before any resulting decreases in $K^k$ surface levels were seen. The heterogeneous downregulation of $K^k$ observed in PBLs and splenocytes is likely a reflection of different regulatory mechanisms for class I expression amongst the different leukocyte subpopulations (263). The higher capacity for class I expression demonstrated by the identified murine T cell subset could be a reflection of their activation state or have other functional implications.

It is likely that $K^k$ down-regulation is directly caused by $K^b$ transgene overexpression, which could conceivably lead to increased competition for nuclear transcription factors, ER chaperones, $\beta_2$-microglobulin, access to the TAP complex, peptide, transport components, and other shared resources. The differences observed between wild-type and cytoplasmic mutant $K^b$ can have several interpretations. The first and simplest explanation is that the differences are a reflection of cell surface internalization rates alone. Under this model, $K^b$ mutant molecules would reach a higher steady-state surface level than $K^bWT$ molecules when expressed at equal transgene dosage levels. Thus, similar levels of $K^k$ down-regulation would reflect similar amounts of mutant and wild-type transgene-encoded protein production. This explanation is supported by the endocytosis experiments, which showed clearly that the mutant $K^b$ molecules are deficient in their ability to undergo constitutive internalization in activated T cells. Other previous studies have also highlighted the importance of cytoplasmic tail
determinants in cell surface internalization. While it seems likely that this explanation is sufficient to explain the differences observed, splenocyte pulse-chase experiments failed to show a significant difference in the conformational half-lives of mutant and wild-type K\textsuperscript{b} molecules.

An alternate explanation is that “quality control” mechanisms in the ER normally limit the number of class I molecules transported to the cell surface but mutant K\textsuperscript{b} molecules can somehow bypass this restriction. This explanation is supported by the observation that in splenocytes and PBLs, K\textsuperscript{b} wild-type surface expression reached an upper limit which remained relatively unchanged despite increasing amounts of K\textsuperscript{b} transgene dosage (as indicated by increasing K\textsuperscript{k} downregulation, Figures 4-4, 4-5, and 4-6). In contrast, the surface expression of mutant K\textsuperscript{b} molecules continued to increase with increasing transgene dosage, especially in the case of the ΔVII- and ΔYSTSS-expressing, doubly transgenic mice. This idea is further supported by $[^{35}\text{S}]$ pulse-chase experiments, which demonstrated that K\textsuperscript{b}WT molecules were able to delay the maturation of K\textsuperscript{k} molecules much more effectively than ΔYSTSS and other mutant molecules, despite the fact that the synthesis rates of the K\textsuperscript{b} mutants were significantly higher (Figure 4-9). The co-immunoprecipitation of high molecular weight bands with K\textsuperscript{k} in only the K\textsuperscript{b}WT-expressing transgenics may imply that K\textsuperscript{k} biosynthesis is slowed to the point where interactions with high molecular weight complexes, perhaps in the ER, are stabilized. The appearance of lower molecular weight bands in these IPs (especially after a two hour chase) is suggestive of other stabilized molecular interactions, possibly with chaperones or peptide-loading components. Alternatively, these lower molecular weight
bands may represent K\textsuperscript{k} degradation products. These data taken together may imply that wild-type and mutant molecules do not traverse exactly the same path to the cell surface. Phosphorylation of the cytoplasmic tail could potentially mediate some of the interactions between class I and molecules known to be important for its maturation and oligomerization and may thus act as a quality control mechanism governing ER egress.

A third possible explanation is the existence of a cell surface "quantity control" mechanism mediated by kinases and phosphatases and executed by clathrin components which strictly controls the levels of total class I molecules in lymphocytes. This would allow for fine-tuning of class I surface levels, and could be exploited to adapt to changing conditions where higher levels of class I expression are required. K\textsuperscript{b} mutant molecules, under this model, would not be subjected to the same control as wild-type molecules, allowing them to build up to abnormally high cell surface levels. These models will be discussed in greater detail in Chapter 6.

Interpreting the results from the ΔY and ΔVII mice is relatively straightforward due to their K\textsuperscript{b} molecules running at the predicted molecular weights on SDS-PAGE. However, some caution must be used when interpreting the results from the ΔSTSS and ΔYSTSS mice, whose K\textsuperscript{b} protein products showed two isoforms that were independent of glycosylation state. The less predominant isoform ran at the expected molecular weight (~44.5 kD) but the majority of the labeled K\textsuperscript{b} pool ran at a significantly lower MW (~42.5 kD). As discussed in Chapter 3, several explanations could account for this phenomenon.
The first explanation involves alternative mRNA splicing. Class I molecules are known to undergo natural alternative mRNA splicing in some cell types which often splices out exon VII. Very little is known about the mechanism which mediates this splicing, although a single intron nucleotide has been shown to be responsible for differential use of exon VIII in H-2K molecules (264). It is possible that the mutations introduced into the exon VII coding region created an alternative splicing site which led to the generation of the lower MW form seen on SDS-PAGE. However, a re-examination of the sequence data for these clones and sequence analyses by several splice site recognition computer programs have failed to indicate the potential creation of such a site. Sequencing of splenocyte K\(^b\) mRNA should resolve these questions.

Antiserum raised against the cytoplasmic protein domain encoded by exon VIII (249) was able to immunoprecipitate both isoforms of these K\(^b\) mutants (data not shown). Exon VII-specific antiserum, however, failed to recognize either mutant isoform and furthermore gave a very weak signal for wild-type K\(^b\). This lack of K\(^b\) mutant recognition is difficult to interpret, because it could be due either to the lack of exon VII or to lack of recognition of the exon VII domain due to the four amino acid changes. The raising of antisera against a ΔSTSS-specific peptide would likely provide a resolution to some of these questions.

There are several alternative explanations that could account for the presence of the K\(^b\) mutant isoforms. For instance, the presence of non-phosphorylatable class I on the cell
surface may potentially generate a feedback signal that activates class I alternative mRNA splicing mechanisms. Also, because these experiments were performed on bulk splenocytes, it is possible that the two observed isoforms are cell type-specific. Mature wild-type K\textsuperscript{b} molecules showed only a single isoform in splenocyte labelings, so point mutations to exon VII may possibly lead to B cell- or T cell-specific alternative splicing.

Finally, post-translational modifications other than glycosylation may explain the presence of the two isoforms. Lack of phosphorylation in the K\textsuperscript{b} mutants leading to cytoplasmic conformational changes and a lower apparent MW on SDS-PAGE is a possibility, albeit an unlikely one. This is because the nature of the mutations is such that \textit{no} phosphorylation should be possible. If this were the case, one would expect to see one isoform, not two. Other cytoplasmic modifications have been proposed for class I molecules, including the recent identification of a largely uncharacterized acidic modification mapping to the exon VI & VII region (255). Interestingly, computer motif searches have shown the amino acid sequence for exon VII also includes a potential myristylation site. As such, the two isoforms may eventually be explained by an uncharacterized cytoplasmic modification that can be altered by amino acid substitution of the conserved serine and threonine residues.

While some of the details regarding the cytoplasmic tail’s role in expression and trafficking have been uncovered, the functional relevance for MHC class I molecules has remained unclear. Maintaining a strict steady-state level of surface class I in lymphocytes may be important for natural killer (NK) cell surveillance and recognition,
since NK cells are known to target cells that are deficient in class I expression (265). It is still not known why activated T cells rapidly internalize and recycle their class I molecules or whether this has any significance for antigen presentation. Recently, a conserved tyrosine residue in the cytoplasmic tail of CD1b and CD1d was shown to be essential for their internalization and recycling through endosomal compartments where these non-classical class I molecules acquire glycolipid antigens for presentation (177)(237)(238). The next chapter examines whether mutation of the tyrosine-based endocytic motif or of the adjacent phosphorylation sites of class I molecules has any functional consequences for K\textsuperscript{b}-restricted antigen presentation \textit{in vivo}. 
Chapter 5 - Antigen Presentation by H-2 K\textsuperscript{b} Cytoplasmic Tail Mutants

5.1 Introduction

While a number of studies have demonstrated that the MHC class I cytoplasmic domain has a role in intracellular trafficking (188, 208, 220), it has not been clearly established whether antigen presentation function is affected by cytoplasmic determinants. Two previous studies have examined antigen presentation in L cells transfected with wild-type and cytoplasmic domain-deleted H-2L\textsuperscript{d}. While both groups found that allo-killing and L\textsuperscript{d}-restricted presentation of an influenza virus epitope could be mediated effectively by the cytoplasmic truncatants, one group observed a substantial decrease in their ability to present vesicular stomatitis virus (VSV)-derived antigens to specific CTL (215). L cells transfected with truncated L\textsuperscript{d} are killed by VSV-specific CTL following VSV infection only 20% as efficiently as L\textsuperscript{d} wild-type transfectants. The results of Zuniga et al. directly contradicted this data, reporting no difference between mutant and wild-type L\textsuperscript{d}-restricted VSV antigen presentation (214). The issue has remained unresolved, as no further work has been described on these systems to date.

A number of transgenic mouse systems have been used to assess the role of extracellular MHC class I domains in the development of CTL responses \textit{in vivo}. For example, point mutations to the peptide-binding domain of K\textsuperscript{b} have been demonstrated to have deleterious effects on the generation of K\textsuperscript{b}-restricted specific CTL in transgenic mice.
Likewise, point mutations to extracellular glycosylation sites have been reported to affect the generation of CTL responses, although this is the subject of some debate. Only one transgenic mouse system has been described that assesses the role of the carboxyl terminal of MHC Class I in the development of CTL responses. These transgenic mice expressed gene constructs encoding the extracellular region of H-2D$^b$ but with a GPI anchor-encoding sequence replacing the normal D$^b$ transmembrane and cytoplasmic regions. Compared to wild-type D$^b$-expressing mice, the mice expressing D$^b$-GPI fusion constructs fail to generate an effective CTL response to a D$^b$-restricted immunodominant influenza A virus epitope (235).

The work presented in this chapter builds on these results and attempts to resolve some of the questions concerning the role of the cytoplasmic domain in functional antigen presentation. It brings together the L cell system described in Chapter 3 and the transgenic mouse system described in Chapter 4 for the purposes of examining and comparing K$^b$-restricted VSV antigen presentation both in vitro and in vivo.

VSV is an RNA virus in the genus Vesiculovirus, family Rhabdoviridae (see Figure 5-1) (266). It is a sporadic, re-emerging pathogen that infects cattle, horses, swine and other domestic and wild mammals. In domesticated farm animals, the symptoms are similar to, but less severe than foot-and-mouth disease (267). Although VSV infection is often sub-clinical, and rarely lethal, it has been estimated to result in losses as high as $50 million per year to the livestock industry (268). How VSV spreads is unknown at this time, but insect vectors, mechanical transmission, and movement of animals may be responsible.
Figure 5-1: Vesicular stomatitis virus.

*Rhabdoviridae Vesiculovirus*, Indiana strain. Virions are bullet-shaped with characteristically spiky envelope surface projections. Dimensions: 45-100 nm diameter; 100-430 nm length. Genome: ~11200 nt of single-stranded RNA. The bar represents 100 nm. 125,000x magnification. Micrograph courtesy of Dr. Frank Fenner, John Curtin School of Medical Research, Australian National University, Canberra, Australia.
VSV was chosen as the preferred vehicle with which to examine antigen presentation for a number of reasons. First, it has been well-established that VSV-infected H-2^b mice can present and generate CTL responses against the specific K^b-restricted peptide epitope RGYVYQGL, which is derived from the viral nucleocapsid (N) protein (amino acids 52-59) (50). The epitope is immunodominant in that >95% of VSV-specific CTL derived from these mice recognize and kill targets expressing this epitope on the cell surface (48). Secondly, H-2^k mice do not generate CTL responses to VSV infection (270). This is significant because both the L cell transfectants and the transgenic mice have an H-2^k MHC background. Using the VSV system ensured that any killing by specific CTL was K^b-restricted. Lastly, the contradictory results reported in refs. 214 and 215 were based on VSV experiments performed on transfected L cells. Therefore, one of the goals of this chapter was to attempt to resolve this discrepancy by re-examining VSV presentation in L cells transfected with K^b cytoplasmic mutants.

The first part of Chapter 5 describes these experiments, which characterize the abilities of the K^b-transfected L cells to be sensitized to killing by VSV-specific, K^b-restricted CTL following either pulsing with VSV N-peptide (52-59) or infection with VSV. Presentation was characterized in detail by varying the VSV MOI as well as viral
incubation times. Interestingly, the results indicated that both previous reports were likely correct, and that the discrepancies observed probably arose due to differences in experimental conditions.

The second part of Chapter 5 describes experiments that assess the CTL responses generated against VSV in the K\textsuperscript{b}-expressing transgenic mice. These experiments provide perhaps the most interesting and provocative results of this thesis. Although more work remains to be done to fully characterize the phenomenon, it appears that the conserved tyrosine residue in the cytoplasmic tail of K\textsuperscript{b} is essential for the generation of K\textsuperscript{b}-restricted CTL responses \textit{in vivo}, at least those raised against VSV.

\section*{5.2 Materials and Methods}

\subsection*{5.2.1 Sensitization of L cell fibroblasts to killing by specific CTL}

Transfected L cells were grown to 50\% confluence on 60 mm tissue culture dishes in DMEM supplemented with 2 mM L-glutamine and 10\% FCS and buffered with 10 mM HEPES. VSV was added to the cells in 2 mL of medium at MOIs ranging from 0.2:1 to 100:1 and cell viability was monitored over 24 hours using trypan blue exclusion staining. From this, it was determined that MOIs between 0.5:1 and 10:1 were optimal for maintaining cell viability for a long enough period (>16 hours) to sensitively assay antigen presentation of viral peptides by H-2K\textsuperscript{b}. Cells were infected with VSV at MOIs of 1:1, 3:1, and 10:1 for various incubation periods before exposing the cells to VSV-specific CTLs (assay described in the following sections). L cells were also sensitized to
CTL killing by pulsing $1 \times 10^6$ fibroblasts in 200 µl medium with 1 uM VSV N-peptide (52-59) at 37°C for 2 hours.

5.2.2 Viral Infection of mice and CTL culture

Mice were infected by intraperitoneal injection of 100 to 400 µl VSV preparation titrated to $10^8$ virus particles per mL. Mice were maintained in clean, individually isolated HEPA-filtered cages for 5 days before sacrificing both infected and naive control mice with CO₂ and removing spleens using sterile technique. Spleens were weighed and kept in ice cold DMEM for up to two hours before Dounce homogenization (<10 strokes) to release splenocytes. Splenocytes were centrifuged at 1500 RPM (Beckman GP) for 10 minutes and washed in cold DMEM three times before counting cells using a hemocytometer. For the purposes of counting, splenocyte aliquots were mixed with 3% acetic acid (to lyse red blood cells) and trypan blue (to stain dead cells). Splenocytes were cultured at 37°C in 10 mL tissue culture flasks at $2.5 \times 10^6$/mL in RPMI complete media supplemented with 1 µM VSV N-peptide (52-59).

5.2.3 CTL Chromium release assay

Following 5-6 days of culture with VSV N-peptide (52-59), splenocytes were resuspended in 0.83% ammonium chloride for 3 minutes to lyse remaining red blood cells and then washed three times in warm DMEM. Splenocytes (containing a significant proportion of specific CTL) were counted and diluted to $3.6 \times 10^6$/mL in warm RPMI complete media before aliquoting 150 µl into the top well of a 96-well V-bottom plate. With all the lower wells containing 100 µl of RPMI, 50 µl was removed from the top well and mixed with the 100 ul media from the next lowest well, from which 50 µl was
removed and added to the next well down, and so on. In this fashion, serial dilutions of CTLs were accomplished with each step-wise dilution containing 3x fewer CTL than the previous one.

L cell fibroblasts were sensitized to killing by specific CTL using a variety of methods (outlined above). All target cells were labeled by incubating in $^{51}$Cr (100 μCi per $10^6$ cells in 200 μl DMEM) at 37°C for 2 hours. L cell targets were washed three times in DMEM before counting and resuspending in warm RPMI at a concentration of 40,000 cells per mL. 100 μl of $^{51}$Cr-labeled cells were added to each well of the 96-well plates containing the CTLs. Thus, 4,000 target cells per well resulted in effector:target (E:T) ratios of 90, 30, 10, 3, 1, 0.3, 0.1, and 0.03 to one. Plates were centrifuged at 800 RPM (Beckman GP) for 4 minutes followed by incubation at 37°C for 4 hours to allow CTL killing of target cells. Plates were spun down again and 100 μl of supernatant was collected from each well of the 96-well plate and counted in a gamma counter. The amount of $^{51}$Cr released by the target cells and detected in the supernatant is proportional to the amount of CTL killing. Spontaneous $^{51}$Cr release by labeled cells was measured in parallel by also incubating 4000 target cells in 200 μl RPMI complete (in the absence of CTL) for 4 hours at 37°C. Maximum release of $^{51}$Cr was quantified by lysing 4,000 target cells in 2.5% Triton X-100. In all experiments shown, spontaneous $^{51}$Cr release was less than 15% of maximal $^{51}$Cr release. All E:T combinations were performed in triplicate and specific $^{51}$Cr release was calculated using the following formula:

\[
\% \text{ Specific } ^{51}\text{Cr Release} = \frac{\text{Experimental release} - \text{Spontaneous release}}{\text{Maximum release} - \text{Spontaneous release}} \times 100\%
\]
5.3 Results

PART I - L cell transfectants

5.3.1 Peptide-pulsed L cells as targets for specific CTL

L cell transfectants were assayed for their abilities to act as targets for specific CTL following incubation with VSV N-peptide (52-59). Both high- and low-expressing clones from each K\textsuperscript{b} variant were \textsuperscript{51}Cr labeled and pulsed with 1 \textmu{}M peptide for two hours before exposure to VSV-specific CTL at various effector:target ratios. The CTL were derived from the spleens of VSV-infected C57/BL6 mice and restimulated in culture as described in Materials and Methods. The results are summarized in Figure 5-2.

All transfectants were effectively sensitized to killing by peptide pulsing, and although there was a range of killing efficiencies seen, there were no obvious differences observed between K\textsuperscript{b}WT and K\textsuperscript{b} mutant-expressing clones. A rough correlation (seven of ten clones) was observed, however, between K\textsuperscript{b} surface expression level and efficiency of killing by specific CTL (data not shown).

5.3.2 VSV-infected L cells as targets for specific CTL

The next set of experiments assayed the L cell transfectants' capabilities to act as targets for specific CTL following VSV infection. Two K\textsuperscript{b}WT clones and two ΔYSTSS clones (high and low expressors from each group) were used for the initial experiments. L cells
were infected with VSV at MOIs of 1, 3, and 10 to one and were subsequently exposed to VSV-specific CTL 10 hours post-infection. The CTL assay results, shown in Figure 5-3, indicated that there was significantly less specific $^{51}$Cr release from the L cells expressing the ΔYSTSS mutant compared to the K$b$WT-expressing clones. This phenomenon was observed at all three MOIs, with the greatest difference between wild-type and mutant clones occurring at an MOI of three to one. At this MOI, the ΔYSTSS clones released an average of 56% less $^{51}$Cr than the K$b$WT clones (30:1 E:T ratio).

Repeating the CTL experiment at 15 hours post-infection significantly decreased the differences observed between K$b$WT and ΔYSTSS clones compared to the killing seen at 10 hours post-infection. Mutant clones in this experiment released an average of 19% less $^{51}$Cr than K$b$WT clones at a 3:1 MOI, 10:1 E:T ratio (data not shown).
Figure 5-2: Peptide-pulsed transfectants as targets for specific CTL.

Fibroblasts expressing comparable levels of surface K^b were ^51^Cr-labeled and pulsed for 2 hours with 1 \( \mu \)M VSV N-peptide (RGVYQGL) before exposure to VSV-specific CTL at various effector:target (E:T) cell ratios. Specific ^51^Cr release is proportional to the amount of VSV-specific CTL killing. Data shows means of three replicate experiments. Error bars represent standard error measurements.
Figure 5-3: VSV-infected transfectants as targets for specific CTL.

(next page) Fibroblasts expressing comparable levels of surface K^b were infected with VSV for 10 hours at MOIs of (A) 3:1 or (B) 10:1, ^51Cr-labeled, and exposed to VSV-specific CTL in a standard ^51Cr release assay. Data represents means of triplicate data points from two replicate experiments. Error bars represent standard error measurements.
5.3.3 Time course of VSV infection

Since the initial set of CTL experiments indicated that viral incubation time may be an important component of VSV presentation and subsequent CTL recognition, a more detailed study of this phenomenon was undertaken. Five L cell lines (one K\textsuperscript{b}WT and one of each of the four mutants) expressing similar levels of surface K\textsuperscript{b} were selected to undergo a time course of VSV infection followed by \(^{51}\text{Cr}\) labeling and incubation with VSV-specific CTL. Parallel plates of cells were infected at an MOI of 3:1 every three to four hours such that viral incubation times of 3, 6, 9, 12, and 16 hours could be assayed for each cell line simultaneously. The results are shown in Figure 5-4.

The data showed that the K\textsuperscript{b}WT clone was the most sensitive to killing by VSV-specific CTL, measuring the highest levels of specific \(^{51}\text{Cr}\) release in the overall experiment. The time course also revealed that the K\textsuperscript{b}WT clone acquired this sensitivity at a faster rate compared with the mutant clones, as shown in Figure 5-5. At an E:T ratio of 30:1, the K\textsuperscript{b}WT clone achieved 40% specific \(^{51}\text{Cr}\) release an average of 8.3 h post-infection, whereas the ΔY clone averaged 10.2 h, the ΔVII clone 11.3 h, the ΔSTSS clone 15.1 h, and the ΔYSTSS clone 16.0 h to reach this level of killing. Figure 5-4B shows that the K\textsuperscript{b}WT clone acquired most of its CTL sensitivity between 6 and 12 hours post-infection, followed by a plateau between 12 and 16 hours. The ΔY and ΔVII clones showed a similar trend, although the slopes of \(^{51}\text{Cr}\) release between 6 and 12 hours (indicating rate of acquisition of CTL sensitivity) were not as steep as for the K\textsuperscript{b}WT clone (4.7% and
Figure 5-4: VSV infection time course and CTL killing assay.

Fibroblasts expressing comparable levels of surface $K^b$ were infected with VSV (3:1 MOI) for 3, 6, 9, 12, or 16 hours, $^{51}$Cr-labeled, and exposed to VSV-specific CTL at various effector:target (E:T) cell ratios. Data points show the means of triplicate experiments with error bars representing standard error.
5.3% per hour, respectively, compared to 7.5% per hour for K\textsuperscript{bWT}). The ΔSTSS and ΔYSTSS clones, by contrast, were much more significantly delayed in their acquisition of CTL sensitivity between 6 and 12 hours post-infection (3.2 % and 2.2% per hour, respectively). From 12 to 16 hours, however, these clones "caught up" somewhat to the others by demonstrating a significant increase in their rates of acquisition of sensitivity to killing (up to 4.7% and 5.8% per hour, respectively, between hours 12 and 16). Similar results were observed upon repeating this experiment using a different set of five clones (one K\textsuperscript{bWT} and four mutants). At time points later than 16 hours p.i., it became difficult to assay specific \textsuperscript{51}Cr release due to the large increase in background \textsuperscript{51}Cr release from control cells and decreased cell viability (likely due to viral-induced lysis.)
Figure 5-5: Comparison of antigen presentation in L cell transfectants.

Graph showing L cell transfectants’ acquisition of sensitivity to killing by VSV-specific CTL following VSV infection over incubation times from 3 to 16 hours. VSV infections were done at an MOI of 3:1 and data shown was derived from a standard $^{51}$Cr release assay (30:1 E:T ratio). Data represents means of three experiments, with error bars representing standard error.
PART II - Transgenic Mice

5.3.4 Spleens and splenocyte cultures

Transgenic mice were infected with VSV by intraperitoneal injection (1 x 10^7 virus particles per mouse) and were sacrificed 5-6 days later using CO₂ euthanasia. Spleens were removed, weighed, Dounce homogenized, and cultured at 2.5x10^6 splenocytes/mL in RPMI complete medium. A consistent observation was an obvious difference in spleen size amongst the various transgenic mice (Table 5-1). Naive control mice had the smallest spleens overall (average weight 0.16 ± 0.02 g), while VSV-infected ΔY and ΔYSTSS spleens were only slightly enlarged (0.19 ± 0.02 g and 0.21 ± 0.03 g). In contrast, spleens from infected K^bWT transgenic mice were significantly enlarged, averaging 0.34 ± 0.04 g. Spleens from ΔVII and ΔSTSS mice were larger still, weighing an average of 0.53 ± 0.05 g and 0.49 ± 0.05 g, respectively. The spleen weights correlated well with the number of splenocytes present within the spleens (Figure 5-6). This trend was confirmed in multiple experiments and was consistently observed for transgenic mice expressing similar levels of surface K^b. In addition, higher K^b expression correlated well with increased spleen size and splenocyte number.

Another consistent observation was the phenomenon of large-scale clumping of splenocytes within the first 24-48 hours in culture with VSV peptide. While all cultures demonstrated clumping to some degree, the splenocytes from the K^b mutant mice nearly always showed the largest and most numerous clumps. This difference was easily seen
### Table 5-1: Spleen characterization following VSV infection.

Comparison of spleen weights and splenocyte clumping in culture following VSV infection of transgenic and control mice. Mice were injected intraperitoneally with VSV (1 x 10^7 infectious particles per mouse) and five days later spleens were removed, weighed, and splenocytes cultured with 1 μM VSV N-peptide (52-59). Spleen weights and increases listed represent averages from at least three separate VSV experiments. Clumping scores are a purely subjective rating, with 0 representing the lowest degree of clumping seen and 5 representing the highest degree of clumping observed.

* ΔY splenocytes generally demonstrated relatively low levels of clumping in culture following VSV infection, with the exception of a high-dose VSV experiment where extremely high levels of clumping was observed and later associated with a high degree of splenic neutrophilia.

<table>
<thead>
<tr>
<th>SPLEEN</th>
<th>Mean Weight</th>
<th>Avg. Increase</th>
<th>Clumping Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57 naive</td>
<td>0.14 g</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>C57 VSV inf.</td>
<td>0.25 g</td>
<td>79 +/- 18%</td>
<td>2</td>
</tr>
<tr>
<td>KbWT (hi exp.) naive</td>
<td>0.18 g</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>KbWT (hi exp.) VSV inf.</td>
<td>0.36 g</td>
<td>100 +/- 14%</td>
<td>2</td>
</tr>
<tr>
<td>VII - naive</td>
<td>0.18 g</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>VII - VSV inf.</td>
<td>0.51 g</td>
<td>183 +/- 27%</td>
<td>5</td>
</tr>
<tr>
<td>STSS (hi exp.) naive</td>
<td>0.18 g</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>STSS (hi exp.) VSV inf.</td>
<td>0.55 g</td>
<td>205 +/- 31%</td>
<td>5</td>
</tr>
<tr>
<td>Y naïve</td>
<td>0.17 g</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Y - VSV inf.</td>
<td>0.21 g</td>
<td>24 +/- 11%</td>
<td>1/5*</td>
</tr>
<tr>
<td>YSTSS naïve</td>
<td>0.14 g</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>YSTSS - VSV inf.</td>
<td>0.20 g</td>
<td>42 +/- 17%</td>
<td>3</td>
</tr>
</tbody>
</table>
Figure 5-6: Splenocyte numbers following VSV infection.

Mice were injected intraperitoneally with VSV ($1 \times 10^7$ infectious particles per mouse) and spleens were removed five days later. Splenocytes were routinely counted before culturing cells for VSV CTL experiments. The bar graphs represent mean splenocyte counts derived from a minimum of three separate experiments. Error bars represent standard error measurements.
with the naked eye. Microscopic observation revealed that the cell clumps seen in the mutant splenocyte cultures contained several dozens of cells tightly associated in grape-like clusters that were largely non-adherent. Smaller degrees of cell clustering were observed in the $K^b$WT and C57/BL splenocytes, with individual clumps typically containing fewer than a dozen cells. The clusters generally broke apart over the third to fourth day in culture. No further quantitation was made of this phenomenon.

5.3.5 CTL responses of mice to VSV infection

Transgenic mice demonstrating similar $K^b$ expression levels were assayed for their abilities to generate a $K^b$-restricted CTL response to VSV infection. Splenocytes were cultured for 5 days with 1 μM VSV peptide, washed, and exposed to $^{51}$Cr-labeled, VSV peptide-pulsed L cells expressing the wild-type $K^b$ gene at various effector:target cell ratios. The results, shown in Figures 5-7A and B, demonstrated that ΔSTSS and ΔVII transgenic mice consistently produced 3- to 4-fold more specific CTL than $K^b$WT transgenics. C57/BL control mice usually generated numbers of specific CTL comparable to that of $K^b$WT transgenics or slightly lower. Surprisingly, ΔY mice produced very few specific CTL, with $^{51}$Cr release levels only slightly higher than those observed for naive control mice. Mice expressing the ΔYSTSS transgene demonstrated numbers of VSV-specific CTL approximately 10-fold less than that observed for $K^b$WT mice, but significantly elevated compared to ΔY mice. Specific CTL comparisons are based on the E:T ratios required to achieve a specific level of $^{51}$Cr release from labeled target cells.
Figure 5-7: CTL responses of transgenic mice to VSV infection.

(next page) Mice expressing similar lymphocyte Kb surface levels were injected intraperitoneally with VSV \(1 \times 10^7\) infectious particles per mouse) and five days later spleens were removed. Splenocytes were restimulated in culture with 1 μM VSV N-peptide (52-59) for five to six days before using the bulk CTL in a standard \(^{51}\)Cr release assay. Target cells consisted of \(^{51}\)Cr-labeled, KbWT-transfected L cell fibroblasts that were pulsed for 2 hours with 1 μM VSV N-peptide (RGVYQGL). (top) \(^{51}\)Cr release assay results for naïve and VSV-infected KbWT, ΔVII, and ΔSTSS transgenic mice (bottom) \(^{51}\)Cr release assay results for VSV-infected C57, KbWT, ΔY, and ΔSTSS transgenic mice. The results show the means of triplicate samples and are representative of three separate experiments.
5.3.6 Splenocyte subpopulations following VSV infection

To explore whether ΔY mice could be induced to make a CTL response to a higher dose of VSV, experiments were performed using 4 X 10^7 virus particles per mouse. Results of the ^51Cr release assay were similar to those obtained using a lower dose of virus (data not shown). However, spleens from the ΔY and ΔYSTSS mice were considerably enlarged and splenocytes cultured from these mice (particularly the ΔY mice) demonstrated a high degree of clumping within 3 hours of culture. Although the clumping phenomenon had been frequently observed previously in transgenic splenocyte cultures, the speed at which the clumps formed was unusually rapid.

To identify the cell types that made up the splenocyte populations, a labeling of the splenocytes was performed by staining for surface markers CD4, CD8, B220, and Mac-1. These markers, expressed by T helper cells, cytotoxic T cells, B cells, and macrophages, respectively, normally stain 85-90% of a murine splenocyte population. The results, shown in Table 5-2, indicate a significant deviation from this norm, particularly in the ΔY and ΔYSTSS transgenic mice. In the ΔY mice, only 48% of the splenocytes stained positive for the above markers, whereas in ΔYSTSS mice only 63% stained positive. K^bWT transgenic splenocytes stained 88% positive, similar to naive and VSV-infected C57/BL mice (96% and 92%, respectively).

Intriguingly, both ΔY and ΔYSTSS mice demonstrated K^b-negative cell populations that made up 50% and 35% of their respective splenocyte populations. This was very unusual
### Table 5-2: Splenocyte subpopulations before and after high-dose VSV infection.

Mice were injected intraperitoneally with VSV (4 x 10^7 infectious particles per mouse) and spleens were removed on day 6. Splenocytes were cultured overnight in 1 μM VSV N-peptide followed by staining with FITC-conjugated monoclonal antibodies specific for either B220 (a murine B cell marker), CD4, CD8 (T cell markers), or Mac-1 (monocyte/macrophage marker). Stained cells were analyzed by FACScan and percentages of splenocytes staining positive for the specific markers are listed in the above table. The results shown are from a single experiment.

<table>
<thead>
<tr>
<th>MOUSE</th>
<th>B220</th>
<th>CD4</th>
<th>CD8</th>
<th>Mac-1</th>
<th>TOTAL</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57 (naïve)</td>
<td>61</td>
<td>20</td>
<td>11</td>
<td>4</td>
<td>96%</td>
</tr>
<tr>
<td>C57 (VSV)</td>
<td>58</td>
<td>20</td>
<td>12</td>
<td>4</td>
<td>94%</td>
</tr>
<tr>
<td>KbWT (naïve)</td>
<td>55</td>
<td>18</td>
<td>13</td>
<td>3</td>
<td>89%</td>
</tr>
<tr>
<td>KbWT (VSV)</td>
<td>40</td>
<td>23</td>
<td>18</td>
<td>5</td>
<td>88%</td>
</tr>
<tr>
<td>C3H (naïve)</td>
<td>64</td>
<td>18</td>
<td>9</td>
<td>6</td>
<td>97%</td>
</tr>
<tr>
<td>C3H (VSV)</td>
<td>53</td>
<td>24</td>
<td>11</td>
<td>5</td>
<td>93%</td>
</tr>
<tr>
<td>Y (naïve)</td>
<td>52</td>
<td>21</td>
<td>11</td>
<td>4</td>
<td>88%</td>
</tr>
<tr>
<td>Y (VSV)</td>
<td>16</td>
<td>5</td>
<td>4</td>
<td>14</td>
<td>48%</td>
</tr>
<tr>
<td>YSTSS (naïve)</td>
<td>62</td>
<td>19</td>
<td>9</td>
<td>3</td>
<td>93%</td>
</tr>
<tr>
<td>YSTSS (VSV)</td>
<td>40</td>
<td>9</td>
<td>7</td>
<td>6</td>
<td>63%</td>
</tr>
</tbody>
</table>
considering the fact that these same cells stained positive for $K^k$ (data not shown). These cells also showed an altered forward and side scatter compared to lymphocytes (as visualized by FACScan), indicating a larger diameter and increased granulation content. This was consistent with the scatter patterns typically observed for granulocytes.

To further identify this cell population, cytospins, Giemsa staining, and microscopic examinations of the splenocytes were performed. Splenocytes derived from the C57/BL control mice and $K^b$WT mice appeared to stain normally, showing a large proportion of lymphoid cells with characteristically small size, large nuclei, and sparse cytoplasm (see Figure 5-8). By contrast, the ΔY and ΔYSTSS splenocytes showed a relatively high proportion of other cell types present. A pronounced degree of neutrophilia was observed, especially in the ΔY splenocytes, as indicated by the high number of cells containing ring- or horseshoe-shaped nuclei. Erythroblasts and megakaryocytes were also observed in increased numbers among the ΔY and ΔYSTSS splenocytes, in addition to another type of blast cell that was not identified. C3H control mice, ΔVII, and ΔSTSS mice infected with a low or a high dose of VSV did not show any gross splenocyte population aberrations as assayed by FACScan or by cytological examination (data not shown).
Figure 5-8: Mouse splenocytes following high-dose VSV infection.

(next page) Transgenic and control mice were injected intraperitoneally with $4 \times 10^7$ virus particles per mouse and spleens were harvested 6 days later. Splenocytes were cultured for 16 hours before making cytospin slides and staining the cells by Giemsa staining. Splenocytes from the ΔY mouse demonstrated excessive clumping in culture and showed significantly altered splenocyte subpopulations (see Table 5-2). In addition, the ΔY spleen (bottom left panel) showed a high degree of neutrophilia (as indicated by donut- or horseshoe-shaped cell nuclei), and had a significant infiltration of other unidentified blast cells that were large, showed diffuse nuclear staining, and tended to form clumps. The ΔYSTSS mouse showed less neutrophilia but demonstrated other splenocyte aberrations (see text). By contrast, spleens from C57/BL and K$^b$WT transgenic mice demonstrated normal splenocyte subpopulations that consisted of 85-95% B and T lymphocytes (upper two panels). VSV-infected ΔVII and ΔSTSS mice showed similar results to the C57/BL and K$^b$WT mice (data not shown).
5.4 Discussion

5.4.1 VSV Presentation in L cell fibroblasts

CTL experiments were performed in order to compare presentation of a K\textsuperscript{b}-restricted VSV-derived epitope in L cells transfected with either wild-type or cytoplasmic mutant K\textsuperscript{b}. While addition of exogenous VSV N-peptide (52-59) sensitized all of the transfectants to killing by VSV-specific CTL to a similar extent, infection with VSV consistently resulted in more specific \textsuperscript{51}Cr release from K\textsuperscript{b}WT-expressing transfectants. This effect was diminished somewhat with longer viral incubation times and larger MOIs. This result indicated that mutant K\textsuperscript{b} was able to effectively present the immunodominant epitope following VSV infection, but that it may not present it as efficiently as wild-type K\textsuperscript{b}.

To further characterize this observation, CTL time course experiments were performed on the L cell transfectants following infection with VSV. The results showed that K\textsuperscript{b} wild-type transfectants clearly presented the VSV epitope the most efficiently of all the transfectants. They were significantly more sensitized to killing by CTL, particularly at 9 and 12 hours post-infection, than all of the K\textsuperscript{b} mutant transfectants. The ΔSTSS- and ΔYSTSS-expressing transfectants presented the VSV epitope with the lowest efficiency, and the ΔY and ΔVII transfectants demonstrated intermediate presentation efficiencies. While the K\textsuperscript{b}WT transfectants reached a peak of sensitization at approximately 12 hours post-infection followed by a killing plateau, the K\textsuperscript{b} mutant transfectants still showed a
significant increase in VSV presentation from 12 to 16 hours p.i. This “delayed presentation” effect was especially apparent in the ΔSTSS and ΔYSTSS transfectants.

Interestingly, these results are consistent with both previously published reports ((214, 215), discussed in this chapter’s introduction), despite the fact that their results are in direct contradiction to one another. Like Murre et al., we observed a significant difference in the presentation of VSV epitopes in L cells transfected with wild-type and cytoplasmic mutant class I molecules. We also found that the extent of these differences was dependent upon incubation time and viral MOI. Thus, by using a high MOI and/or a longer incubation time, the differences between wild-type and mutant transfectants’ abilities to present VSV epitopes becomes less discernable. In the report by Zuniga et al., the experimental MOI used was 100:1. In our hands, this MOI proved to be inappropriate for antigen presentation studies due to reduced cell viability, reduced adherence to tissue culture plates resulting in cell losses, and unacceptably high background levels of $^{51}$Cr release. However, the high MOI used by Zuniga et al. may explain why a difference in VSV presentation was not observed between the mutant and wild-type transfectants. The MOI used in the Murre et al. report was not made explicit but, based on our results, it is likely to have been significantly lower than 100:1.

Why do cytoplasmically mutated class I molecules appear to present VSV epitopes less efficiently than wild-type class I molecules? One explanation is that they are acquiring peptides less efficiently during their biosynthesis than are wild-type molecules. This would imply that cytoplasmic determinants are required for optimal peptide loading in
the endoplasmic reticulum (ER). Our results are consistent with the notion that phosphorylation may play a role in such a process as both serine phosphorylation point mutants, ΔSTSS and ΔYSTSS, proved to be the least efficient at presenting the VSV epitope. Exon VII deletion and point mutation of the conserved tyrosine residue also reduced presentation efficiency, albeit to a lesser extent. The biosynthesis of MHC molecules and their proper oligomerization is a complicated, multi-step process involving numerous cellular components. It is conceivable that these cytoplasmic determinants may provide a level of quality control throughout, or at some stage of class I maturation. Mutation of these determinants could potentially alter this control and consequently result in altered peptide acquisition capabilities. This may occur independently of phosphorylation since altered cytoplasmic domain folding could also conceivably result from the mutations made.

Based on our results it would be fair to ask, how could modestly more efficient antigen presentation be physiologically significant if the antigen is eventually presented anyway? At least three things should be considered to address this point. The first is that viruses, by their nature, are able to effectively hijack cellular machinery to make large quantities of viral proteins that flood the cell in the hours leading up to death by lysis. This is likely to result in a large influx of proteosome-degraded, virally-derived peptides into the ER. The second point is that MHC Class I molecules are not only essential for presenting viral antigens but are also important for presenting tumor antigens, which are not always likely to be present in such high quantities. It is possible that the overproduction of VSV antigen in our experiments served to mask or underplay more serious deficiencies in the
peptide acquisition capabilities of the K\(^b\) mutants. An inability to effectively present low or modest levels of tumorigenic peptide antigens could potentially have very serious consequences \textit{in vivo}.

The third point relates to the number of specific peptide-MHC complexes required on the target cell surface to trigger CTL lysis, estimated to be only approximately 50 - 200. Regardless of the true number, an experimental limitation of our antigen presentation studies is the fact that this is an all-or-none assay: either the cells are killed if they are above the MHC-peptide threshold limit, or they are not if they are below the limit. Unfortunately, this type of assay doesn’t allow for determination of actual numbers of specific MHC-peptide complexes, which could possibly reveal even greater differences in VSV N-peptide presentation between wild-type and mutant K\(^b\) molecules.

An alternate explanation of the data is that the differences observed between the wild-type and mutant K\(^b\) molecules is due, wholly or in part, to the half-life of the various K\(^b\) proteins on the cell surface. The fibroblast presentation experiments utilized L cell clones that expressed comparable levels of surface K\(^b\). However, as shown in Chapters 3 and 4, equal K\(^b\) cell surface expression in wild-type and mutant clones does not necessarily result from equal K\(^b\) synthesis rates. In fact, the data suggest that if \textit{de novo} K\(^b\) synthesis rates could be made equal between wild-type- and mutant-expressing clones, the K\(^b\) cytoplasmic mutants would always reach higher steady-state surface expression levels. This is presumably due to a lack of recognition of the mutant cytoplasmic domain.
by internalization components, either due to a lack of phosphorylation and/or lack of a normal tyrosine-based endocytic motif.

It is also possible that the delay in sensitization to killing observed in the mutant clones is simply due to a lower $K^b$ synthesis rate, which results in a greater length of time needed to reach the threshold level of surface MHC-peptide complexes required to trigger specific CTL killing. This hypothesis is supported by experiments that demonstrate that high mutant $K^b$-expressing clones acquire killing sensitivity somewhat faster than low-expressing ones (data not shown). However, while repeating the experiments using these clones lessened the differences observed between mutant and wild-type, the low-expressing $K^b$ wild-type clones continued to present the VSV epitope the more efficiently than any of the high-expressing $K^b$ mutant transfectants (see Figure 5-3). Therefore, while different synthesis and internalization rates may explain the data in part, it is not likely to explain the entire phenomenon. Detailed experiments aimed at carefully quantifying $K^b$ synthesis rates, as well as determining the actual number of specific surface MHC-peptide complexes generated in mutant and wild-type clones, are needed to clarify this question further.

### 5.4.2 VSV CTL Response in Transgenic Mice

In contrast to VSV infection of fibroblasts, differences in VSV responses between the $K^b$-expressing transgenic mice proved to be both less subtle and more complex. The complexity was somewhat expected, since in vivo immune responses involve numerous
interactions of various cell types that must occur in a very specific sequence in order to generate specific T cell-mediated immunity. However, in contrast to all of the previous data, the CTL responses to VSV in the transgenic mice implicated the tyrosine residue alone as being the most critical determinant of the cytoplasmic domain. Mice expressing the ΔY mutant showed a complete failure to generate K\(^b\)-restricted CTL responses in response to VSV infection. In contrast, K\(^b\)WT-expressing transgenic mice and C57/BL mice (which express wild-type K\(^b\)) were able to generate regular CTL responses to the same immunodominant epitope. Almost as surprisingly, mice expressing the ΔSTSS and ΔVII mutants consistently generated significantly more vigorous CTL responses compared to K\(^b\)WT-expressing mice, as measured by CTL killing as well as by spleen weight and splenocyte numbers. The ΔYSTSS mice demonstrated an intermediate VSV CTL response phenotype, somewhere in between that of the K\(^b\)WT mice and the ΔY mice.

Due to the aforementioned complexities inherent within an in vivo immune response, interpretation of the transgenic VSV CTL data is a challenging task, and at this stage of the research must remain somewhat speculative in nature. However, similarities with previously studied antigen presentation systems such as MHC Class II/Ii and CD1b and CD1d makes it easier to draw parallels and provide possible models to explain our results.

All of these molecules have been shown to be dependent on cytoplasmic determinants that strongly resemble endocytic motifs for their antigen presentation capabilities.
Mutation of a conserved tyrosine residue in the cytoplasmic tail of CD1b renders this MHC class I-like molecule unable to acquire and present glycolipid antigens to γδ T cells. Similarly, MHC class II molecules and Ii are both dependent on conserved cytoplasmic di-leucine motifs for their abilities to acquire and present protein antigens. Mutations to these endocytic motifs have been demonstrated to limit access of these molecules to endocytic compartments where antigens are normally acquired. Therefore, it is not unique that mutation of a conserved endocytic motif should affect antigen presentation. It is somewhat surprising, however, that Class I-mediated presentation, which unlike the other systems is not normally associated with exogenous antigens and dependent on endosomal trafficking, is so dramatically affected by mutation of a potential tyrosine-based endocytic motif.

Our data has shown that antigen presentation in VSV-infected L cell fibroblasts is only moderately delayed by ΔY mutations, so it is probably unlikely that ΔY fibroblasts in vivo in are failing to present VSV-derived N-peptide. However, the critical first step for the initiation and generation of CTL responses in vivo is antigen presentation by professional APCs, particularly dendritic cells and macrophages. Intriguingly, these cell types are known to be capable of presenting exogenously-derived antigens in the context of MHC class I molecules. It has been debated whether exogenous antigen presentation by class I molecules in professional APCs is, in fact, a crucial early step in initiating most CTL responses. As touched on in the introductory chapter, this would allow CTL reactions to be initiated by professional APCs without the APCs themselves having to undergo viral infections or express tumor antigens endogenously.
The failure of the ΔY transgenic mice to mount an effective CTL response against VSV may be due to ineffective presentation of viral antigens by APCs. The most potent APCs known are dendritic cells, so it will be of great interest to determine whether ΔY-derived dendritic cells are capable of K\(^b\)-restricted exogenous antigen presentation. Preliminary studies have determined that the ΔY mutant \(i\) is expressed on the surface of DCs, but specific antigen presentation studies have yet to be done. If the tyrosine does prove to be a critical residue for giving class I molecules access to endosomal compartments where they acquire exogenous antigens for presentation on the surface of APCs, it would go a long way to explaining its high degree of evolutionary conservation.

Another explanation for the lack of VSV CTL response in the ΔY transgenic mice could be a failure at the T cell level. As alluded to in the introduction, MHC class I molecules are intimately involved in numerous aspects of T cell selection and development. It is possible that the ΔY mutation is adversely affecting intrathymic selection of K\(^b\)-restricted TcR-expressing T cells. This could potentially be due to lack of surface expression of ΔY-K\(^b\) by the thymic epithelium. Alternatively, it could also imply that a specialized MHC class I signaling pathway active in thymic stromal cells normally controls thymic selection by delivering positive or negative signals to maturing T cells in response to low or high avidity MHC binding. Although no evidence exists to support such a pathway, much about the general nature of T cell selection signals remains unclear. Experiments designed to address some of these questions will be discussed in Chapter 6.
It has been previously suggested that because the cytoplasmic domain is encoded by three separate exons, they encode separate functional domains. The natural occurrence of exon VII-deleted splicing isoforms added to this speculation, especially since this exon was known to encode for at least one highly conserved serine phosphorylation site. One of the most surprising aspects of the transgenic mouse VSV CTL data was the wide difference in phenotypes observed between the various K\textsuperscript{b} mutants. Both the ΔSTSS- and ΔVII-expressing mice generated VSV CTL responses consistently exceeding those observed for K\textsuperscript{b}WT-expressing transgenics and C57/BL control mice. This was not simply due to increased K\textsuperscript{b} surface expression (as documented for lymphocytes in Chapter 4), since ΔSTSS-expressing mice with very similar K\textsuperscript{b} surface expression levels to K\textsuperscript{b}WT-expressing mice still generated approximately three times more VSV-specific CTL.

This bulk culture CTL data unfortunately does not give us an accurate indication of the numbers of K\textsuperscript{b}-restricted VSV-specific CTL precursors present in the initial pre-culture splenocyte populations. It is possible that the \textit{in vitro} CTL stimulation step (with VSV peptide) was solely responsible for the increase in active CTL observed for ΔSTSS and ΔVII mutants. However, the obvious differences in the spleen masses and cell counts suggest strongly that VSV-specific CTL precursors were not equal or comparable at the time of splenectomy.

How could deletion of exon VII or point substitution of exon VII phosphorylation sites lead to increased K\textsuperscript{b}-restricted CTL responses \textit{in vivo}? One possibility is that exon VII K\textsuperscript{b}...
mutants are internalized less efficiently from the cell surface of APCs, leading to lengthened antigen presentation times and therefore increased overall CTL stimulation. Perhaps the disengagement of TcR from class I molecules on APCs is normally partially mediated by class I internalization. An internalization defect in Class I may have thus led to the excessive clumping observed in mutant CTL cultures. Another possibility is that lack of phosphorylation somehow results in increased endosomal access, possibly leading to increased levels of class I exogenous antigen presentation in APCs. It would be interesting to determine the levels of exon VII-deleted class I isoforms naturally expressed by DCs and examine whether these isoforms have abilities to present exogenous antigens that is not shared by full-length class I molecules. This could explain the significance of these isoforms in nature, which continues to remain a mystery.

Intriguingly, the ΔYSTSS-expressing mice were able to generate VSV-specific CTL responses, but they were significantly weaker than those of K<sup>b</sup>WT-expressing mice. Even the homozygous ΔYSTSS mice, which expressed twice as much surface K<sup>b</sup> in lymphocytes as the K<sup>b</sup>WT transgenics, typically generated 8-10 times fewer specific CTL than K<sup>b</sup>WT mice. This phenotype is intermediate between the ΔY and ΔSTSS phenotypes, suggesting that the dramatic reduction in CTL response induced by the tyrosine point mutation is rescued somewhat by mutations to the conserved serine phosphorylation site(s). The implications of these results will be discussed further in Chapter 6.
Further evidence of an altered VSV immune response in the ΔY and ΔYSTSS mice came from examination of splenocyte subpopulations following a high-dose VSV infection. Spleens from these mice were considerably enlarged and contained excessive numbers of cells. The ΔY splenocytes demonstrated excessive clumping in culture, and B and T lymphocytes were identified as comprising only 40% of the splenocyte population. Over half of the cells were K\(^b\)-negative, but all expressed endogenously-encoded K\(^k\). The ΔYSTSS mice showed a similar but less severe phenotype. In these splenocytes, lymphocytes made up only about 65% of the population, while approximately one-third of the cells were negative for surface K\(^b\). Cytospins and Giemsa staining showed a very high neutrophil infiltrate in the spleens, along with increased numbers of macrophages, erythroblasts, megakaryocytes, and a large number of unidentified blast cells, none of which (with the exception of macrophages) are normally present as part of murine splenocyte subpopulations. These splenocyte aberrations were not observed in naïve, uninfected ΔY or ΔYSTSS mice, nor following VSV infection of any of the other K\(^b\) transgenics, C57/BL, or C3H control mice.

Considerably more work needs to be done to fully characterize this phenomenon, but a few possibilities will be offered here in an attempt to explain this result. Since the phenotype appears only in response to VSV infection, it is possible that excessive viral replication in the spleen is causing infiltrates of neutrophils and granulocytes, important components of innate immunity. Interestingly, murine spleens do not normally support VSV replication. Natural killer (NK) cells, also important contributors to innate immunity, may also have homed the spleen in an attempt by the immune system to
resolve the viral infection in the absence of a strong VSV CTL response. Experiments are currently underway to test whether the unidentified blast cell type was, in fact, NK cells.

Another possibility is the release of chemokines and cytokines causing aberrant homing of cell types to the spleen that are not normally found there. This may be due to CD4\(^+\) T helper cell responses to the VSV infection. CD4\(^+\) T helper cells are known to release a variety of cytokines and are responsible for the activation of a wide range of cell types. It is conceivable that an overactive CD4\(^+\) T cell response is generated to compensate for the lack of a CD8\(^+\) T cell VSV response, leading to the aberrant splenocyte subpopulation phenotypes observed in the ΔY and ΔYSTSS mice.

It will also be of considerable interest to determine why a large proportion of the aberrant splenocytes were negative for surface K\(^b\) but still expressed K\(^k\). This somewhat bizarre result may help to identify one or more cell types in which Class I surface expression is dependent on the tyrosine residue. Allelic loss of Class I expression is also a hallmark of many tumours.

As a final note, it must be remembered that the work in this chapter examined and characterized only one antigen presentation system, that of the vesicular stomatitis virus. There are numerous other K\(^b\)-restricted epitopes that have been characterized and more are being identified each year. It should be apparent that antigen presentation using these other systems must be examined in detail before any general statements regarding the
role of MHC Class I cytoplasmic determinants in the generation of \emph{in vivo} CTL responses can be made. The next chapter will outline some of these other $K^b$-restricted epitopes in a section on future experimental directions. Chapter 6 will also bring together the work outlined in this and other chapters, and will propose some models in an attempt to explain the data presented.
Chapter 6 – Conclusions

The study of MHC class I cytoplasmic mutants in L cell fibroblasts and transgenic mice has brought to light a number of interesting phenomena. Some of the results were expected, based on knowledge of previous studies of class I molecules. Other results were not anticipated, but could be explained at least in part by previously characterized cellular mechanisms. Still other results were completely unexpected, and may be reflections of uncharacterized intracellular and/or immunological phenomena. Before discussing possible models to explain the data, it is probably useful to draw some general conclusions from the overall work.

6.1 General Conclusions

1. Cytoplasmic mutations to K\textsuperscript{b} do not significantly affect the association with murine β\textsubscript{2}-microglobulin during class I biosynthesis or subsequent surface expression in fibroblasts or splenocytes. Nor do they affect K\textsuperscript{b} maturation rates and conformational half-lives of the molecules in these cells.

2. Cytoplasmic mutations cause increased cell surface expression of K\textsuperscript{b} molecules in L cell fibroblasts and in B and T lymphocytes. All four types of mutations resulted in significantly increased K\textsuperscript{b} surface expression compared to K\textsuperscript{b}WT molecules. Mouse splenocytes derived from ΔVII and ΔYSTSS homozygous transgenic animals demonstrated the highest overall K\textsuperscript{b} surface expression, with the ΔY and ΔSTSS K\textsuperscript{b} mutants showing a slightly less pronounced phenotype.
3. Wild-type K<sup>b</sup> molecules in lymphocytes show an upper limit of surface expression that is significantly lower than surface levels observed for mutant K<sup>b</sup> molecules. Once this upper limit is reached, additional K<sup>b</sup>WT transgenes fail to cause increased K<sup>b</sup> surface expression. This upper limit is similar to the level of total class I expression in non-transgenic control animals.

4. Expression of wild-type K<sup>b</sup> genes affects surface expression of endogenous class I molecules more than expression of mutant K<sup>b</sup> genes. In both transfected fibroblasts and transgenic lymphocytes, expression of endogenous class I alleles K<sup>k</sup> and D<sup>k</sup> were significantly more susceptible to downregulated surface expression when K<sup>b</sup>WT molecules were co-expressed. In these systems, downregulation of endogenous class I molecules correlated well with increasing K<sup>b</sup>WT surface expression, such that total class I levels remained relatively constant. While mutant K<sup>b</sup> genes could downregulate endogenous class I expression, it was only observed at very high K<sup>b</sup> surface expression levels. Moreover, total class I reached significantly higher surface levels compared to systems expressing only wild-type class I molecules. Likely related to this, the expression of K<sup>b</sup>WT molecules in splenocytes was observed to delay the maturation of K<sup>k</sup> molecules much more significantly than expression of similar levels of mutant K<sup>b</sup> molecules.
5. Constitutive internalization of $K^b$ molecules in activated T cells is abrogated by cytoplasmic mutations. All four mutations were shown to have significant effects on class I internalization as visualized by confocal immunofluorescence. Quantitation of internalization demonstrated that while all of the mutants were internalized significantly less than $K^b_{WT}$ or $K^k$ molecules, $\Delta YSTSS K^b$ mutants were internalized the least, followed by the $\Delta VII$, $\Delta Y$, and $\Delta STSS K^b$ mutants.

6. Wild-type $K^b$ and mutant $K^b$ molecules in both fibroblasts and lymphocytes show different correlations between molecular synthesis rates and cell surface expression. In general, if wild-type and mutant molecules are synthesized at comparable rates, the mutant molecules will reach a significantly higher level of cell surface expression. Likewise, if wild-type and mutant molecules are expressed on the cell surface at comparable levels, the synthesis rates of mutant molecules will invariably be less. This phenotype was noted especially for the $\Delta VII$, $\Delta STSS$, and $\Delta YSTSS$ mutants.

7. Mutant $K^b$-expressing fibroblast clones acquire sensitivity to VSV-specific CTL killing more slowly than $K^b_{WT}$-expressing clones following VSV infection. Comparison of fibroblasts with similar levels of $K^b$ surface expression showed that $K^b_{WT}$ clones acquired sensitivity to killing the most rapidly, followed by $\Delta Y$- and $\Delta VII$-expressing clones and, following a significant delay, $\Delta STSS$ and $\Delta YSTSS$ clones.
8. In fibroblasts, K\(^b\) molecules containing cytoplasmic mutations bind more exogenously-added K\(^b\)-specific peptide than do K\(^b\)WT molecules. This was demonstrated directly for the ovalbumin-derived peptide SIINFEKL, and was implied for the VSV N protein-derived peptide RGYVYQGL by the increase in the number of conformationally-dependent K\(^b\) complexes on the cell surface (following peptide addition) compared to that observed for K\(^b\)WT molecules.

9. ΔSTSS mutations result in expression of K\(^b\) isoforms that run at lower-than-expected apparent molecular weights on SDS-PAGE. In fibroblasts, the ΔSTSS and ΔYSTSS mutants both demonstrated a single isoform that ran at apparent MWs almost 2 kD less than expected. The two mutants did run at distinct MWs, with the ΔYSTSS mutant running slightly faster, and the ΔSTSS mutant slightly slower, than the ΔVII K\(^b\) mutant (which contains a deletion of 13 amino acids). The lower molecular weight isoforms were also observed in the transgenic splenocytes expressing these mutants, but the cells also expressed a higher molecular weight isoform that migrated at the expected apparent MW. In splenocytes, the lower MW isoform was consistently two to four times more prevalent than the higher MW isoform.

10. Mutations to the conserved K\(^b\) cytoplasmic tyrosine residue abrogate VSV-specific CTL responses in transgenic mice. Mutating exon VII residues (ΔVII, ΔSTSS) led to enhanced VSV-specific CTL responses compared to K\(^b\)WT-expressing transgenic and C57/BL control mice. ΔYSTSS-expressing mice
demonstrated an intermediate phenotype with respect to VSV-specific CTL responses. These mice showed significantly decreased CTL responses compared to K\textsuperscript{b}WT and C57/BL mice, but also significantly more specific CTL than the ΔY mice.

6.2 Possible Models

There are a number of possible models that can adequately explain most of the data presented in this thesis, but it is a much more challenging task to find a single model to explain all of the data. The fact that cytoplasmic mutations were shown to affect basal class I cell surface expression levels in all cell types examined was consistent with the cytoplasmic domain’s documented role in cell surface internalization. While Exon VII deletions have been previously demonstrated to prevent constitutive class I endocytosis in a T lymphoblastoid cell line, this is the first demonstration of cytoplasmic mutations causing an effect on basal levels of class I expression in all resting T and B lymphocytes. The same mutations also prevented constitutive endocytosis in concanavalin A-activated T cells. The implication from these results is that a similar cytoplasmic tail-dependent internalization mechanism is at work in all types of lymphocytes, only in activated T cells it is considerably upregulated.

The specific nature of the point mutations introduced provides some insight into this class I internalization process. The most surprising result was the dependence of this process on the conserved tyrosine residue found within the exon VI coding region. Whereas a
previous study dismissed exon VI residues as having any important role in T lymphoblastoid class I internalization, our results prove that it clearly does. Class I molecules are known to internalize in activated T cells and macrophages through clathrin-coated vesicles (CCVs). CCV formation and endocytosis have been shown to be dependent on tyrosine-based endocytic motifs located within the cytoplasmic domains of a large number of integral membrane proteins. Therefore, our results strongly suggest that the tyrosine residue within class I molecules is part of a tyrosine-based endocytic motif.

Our results also support a previous report that implicated exon VII residues as being essential for T lymphoblastoid class I internalization. The K\(^b\) exon VII deletion mutant used in our studies was expressed at very high levels in fibroblasts and resting B and T lymphocytes, and could not be internalized effectively in activated T cells. If we ignore the possible existence of different splicing isoforms (these will be discussed later), the results from the ΔSTSS mutant suggest a role for serine phosphorylation in the internalization process. This would not be unprecedented, as several membrane proteins have been shown to be dependent on phosphorylation for their subsequent internalization. The ΔYSTSS mutant was expressed at some of the highest cell surface levels observed in lymphocytes and also showed the least internalization in activated T cells. This phenotype was more pronounced than either the ΔY or ΔSTSS phenotypes, supporting the notion that both the tyrosine and serine residues are critical for recognition of the cytoplasmic domain by internalization components.
A simple model to explain the expression and trafficking data is shown in Figure 6-1. In wild-type class I molecules, phosphorylation of the conserved serine residue(s) leads to a conformational change in the structure of the cytoplasmic domain such that the tyrosine residue is subsequently recognized as an endocytic motif. This motif then binds to adaptor complexes that instigate the formation of CCVs. The impetus for serine phosphorylation may possibly come as a result of class I complex dissociation. Under this model, dissociation of β2m and peptide may lead to a conformational change in the HC intracellular region that allows for kinase-mediated phosphorylation. This idea is attractive as a controlled mechanism for clearance of empty Class I HCs from the cell surface. It may partially explain the importance of the cytoplasmic tail in fibroblasts and resting lymphocytes, but it does not explain the very rapid internalization and recycling observed in activated T cells. MHC complex dissociation would have to occur much too rapidly to result in the level of T cell internalization observed. Perhaps in activated T cells, phosphorylation of Class I molecules is upregulated to the point where conformational tri-molecular complexes, and not just empty HCs, are phosphorylated and subsequently internalized.
**Figure 6-1: Potential mechanism of Class I surface expression control.**

Simple model showing potential control of MHC class I surface expression in fibroblasts and resting lymphocytes. Dissociation of $\beta_2m$ and peptide from the class I heavy chain (HC) leads to recognition and serine phosphorylation by a membrane-associated Ser/Thr kinase (K). This phosphorylation event in turn leads to recognition of the tyrosine-based endocytic motif by internalization components such that the HC is internalized via CCVs to early endosomes. From there, they are either bound to $\beta_2m$ and peptide and recycled to the cell surface or are marked for degradation and transported to lysosomes.
Since phosphorylation of Class I molecules has been shown to be upregulated by phorbol esters in all cell types examined, yet only lymphoblastoid cells and macrophages can rapidly internalize Class I molecules, it argues against a role for phosphorylation-dependent internalization in all cell types. Another interpretation of this data is that phosphorylation is not the only requirement for internalization of Class I molecules, but expression of particular internalization components are also required. Perhaps adaptin proteins expressed normally by resting lymphocytes or fibroblasts are expressed at very low levels or do not recognize phosphorylated Class I molecules very efficiently. Upregulated adaptin expression, post-translational adaptin modifications (ie. phosphorylation), or expression of alternate adaptin components could result in greatly increased recognition efficiency of phosphorylated Class I cytoplasmic domains in activated T cells and/or macrophages.

This model works reasonably well for explaining the results from these and other expression and trafficking studies but needs to be appended in order to explain the CTL results from VSV-infected transgenic mice. While a simple model may suffice for a relatively simple system such as intracellular trafficking, it is not likely to account for a complex in vivo process like the generation of CTLs, which requires the precise orchestration of a multitude of cells, receptors, and cytokine-mediated activation signals, among other things.
Perhaps the most striking thing about the lack of observable VSV-specific CTL responses in the ΔY transgenic mice was the fact that its phenotype differed so dramatically from that of the other cytoplasmic mutants, particularly the ΔVII and ΔSTSS mutants. It has been speculated for years that because the cytoplasmic domain is encoded by three separate exons, each one might code for a distinct functional domain (similar to the class I extracellular region). However, this CTL result represents the only indication amongst all the other data in this thesis that there may be a certain amount of truth to this notion.

The fact that the tyrosine residue seems to be critical \textit{in vivo} for the generation of certain CTL responses makes sense based on findings from a number of other studies. For instance, the extremely high level of conservation between species strongly suggests that it is likely important for some \textit{in vivo} process. Secondly, MHC class I-like molecules CD1b and CD1d have both been shown to be dependent on conserved cytoplasmic tyrosine residues for their abilities to present glycolipid antigens to NK1.1\textsuperscript{+} T cells. Tyrosine point mutations have been shown to restrict their access to endosomal compartments where they acquire antigens for presentation. Third, MHC class II antigen presentation is dependent on cytoplasmic di-leucine motifs present in both class II and the invariant chain (II). Di-leucine motifs, like tyrosine-based endocytic motifs, are recognized by adaptin proteins and are important for CCV formation. Lastly, studies on the HIV-nef protein have demonstrated that the human MHC class I tyrosine residue is critical for the \textit{nef}-induced accumulation of class I molecules into CCVs.
The lack of a CTL response against VSV may indicate a failure by professional antigen presenting cells (APCs) to effectively present the VSV N-peptide in the context of tyrosine-mutated K\(^b\) molecules. The importance of dendritic cells (DCs) in initiating priming of naïve CTLs \textit{in vivo} directly implicates these cells as potential candidates for closer examination in the ΔY transgenic mice. DCs are derived from the same myeloid cell lineage as monocytes and macrophages, cell types that are known to internalize class I molecules rapidly through CCVs. These cells are also among the only cells known to present exogenous antigens in the context of class I molecules. If DCs are also able to internalize and recycle class I molecules, it is possible that this process gives class I molecules access to exogenous antigen peptide-loading compartments like MIICs. It is also feasible that mutations to the class I cytoplasmic tyrosine-based endocytic motif would cause a failure of these molecules to access these compartments and, therefore, manifest itself as a failure to present exogenous antigens for CD8\(^+\) cell priming.

This model works well to explain the lack of VSV-specific CTL responses in the ΔY-expressing transgenic mice. However, the model fails to adequately explain the abilities of the ΔVII- and ΔSTSS-expressing transgenic mice to generate more vigorous CTL responses than K\(^b\)WT-expressing or C57/BL control mice. If internalization and CCV-mediated transport through endosomes is necessary for presentation of exogenous antigens, then why aren’t the exon VII cytoplasmic mutations causing similar defects in antigen presentation? The fact that all four of the cytoplasmic mutations led to a similar inhibition of K\(^b\) internalization in activated T cells makes the CTL results all the more puzzling.
A comprehensive APC model that takes all of the trafficking and CTL data into account should likely contain the following elements:

1. The conserved tyrosine residue is important for allowing Class I molecules to gain access to exogenous peptide-loading compartments (EPLCs). Alternatively, it is important for the transport of Class I molecules from these compartments to the cell surface.

2. Exon VII is not required for Class I molecules to gain access to EPLCs. Exon VII-deleted Class I molecules may have preferential access to these compartments.

3. Serine phosphorylation of the cytoplasmic domain is not required for Class I trafficking through EPLCs. Non-phosphorylated Class I molecules may have preferential access to these compartments.

4. Internalization from the plasma membrane is not the primary means by which Class I molecules gain access to EPLCs. Endocytosis from the plasma membrane is similarly inhibited in all four cytoplasmic mutants.

These basic premises describe a model for professional APCs that explains many of the CTL results observed for the various mutants. The idea that exon VII deletions and non-phosphorylatable class I molecules gain preferential access to EPLCs explains the enhanced CTL responses observed in mice expressing these $K^b$ mutants. It also helps to explain the results observed for the $\DeltaYSTSS$ mice, which showed relatively weak but easily measurable CTL responses to VSV infection. This result suggests that the tyrosine
residue alone is not critical for access to EPLCs and that some of the ΔYSTSS molecules, possibly due to their inability to become phosphorylated, do gain some limited access to these compartments.

The notion that exon VII-deleted splicing variants may gain preferential access to EPLCs suggests a function for this naturally-occurring but enigmatic class I isoform that is distinct from full-length molecules. A distinct function has been speculated for these isoforms for a number of years, but no differences other than a lack of internalization in T lymphoblastoid cells has ever been reported.

As discussed in Chapters 3 and 4, the lower-than-expected molecular weight isoforms observed in the ΔSTSS and ΔYSTSS mutants may represent exon VII-deleted splicing isoforms. If this were the case, the similar CTL and internalization results observed for the ΔVII and ΔSTSS mutants would have a simple explanation. The ΔYSTSS CTL results could also be explained using this reasoning. Deletion of exon VII in these mutants would still leave the tyrosine mutation intact, restricting somewhat but not completely the trafficking of these molecules through EPLCs.

If we examine the murine exon VII splicing isoforms in a little more detail, some interesting things become apparent. First, instead of the tyrosine-based endocytic motif having a ---YALAPGSQ--- amino acid sequence (as do full-length molecules), the exon VII-deleted motif becomes ---YALAPV. This may be significant because not only is the valine from exon VIII well-conserved between species (see Figure 1-8), it is also
hydrophobic. Tyrosine-based endocytic motifs often require a hydrophobic residue at the Y+3 position. While the exon VII-deficient class I splicing isoform contains the hydrophobic valine at the Y+5 position, it may still contribute to a significantly increased degree of recognition as a tyrosine-based motif (see Figure 6-2).

The potential ability of class I molecules to access endosomal compartments and acquire exogenous antigens in macrophages and dendritic cells is not necessarily dependent upon endocytosis of these molecules from the cell surface. Since internalization of all four mutants from the surface of activated T cells was inhibited, it argues against the idea of increased endosomal access by certain of the Kn mutants. It is possible that some class I molecules pass through these compartments en route to the cell surface. There have been reports of invariant chain (Ii) binding to MHC class I proteins, leading to speculation that certain subsets of class I molecules can access MIICs in a manner similar to class II molecules. This trafficking could potentially be mediated by the tyrosine-based motif within exon VI (particularly for exon VII-deleted forms) or alternatively by the potential exon VII leucine-based motif (for non-phosphorylated class I molecules). Once acquiring peptides in the endocytic compartments, they are transported to the cell surface where they presumably stay for an extended period of time due to their lack of internalization.
Figure 6-2: Potential endocytic motif created by exon VII splicing.

Schematic representation of MHC class I cytoplasmic domain before and after splicing out of exon VII. Contiguous exon VI-VIII sequences result in the shift of a hydrophobic valine residue to the Y+5 position, potentially forming a tyrosine-based endocytic motif. The predicted Exon VII splicing products of the murine H-2K\(^b\) and the human HLA-A molecules are shown.
The results showing that maturation of $K^k$ molecules was delayed significantly more by wild-type $K^b$ molecules than by mutant $K^b$ molecules supports this notion. The fact that mutant $K^b$ molecules interfered less with the normal biosynthesis of $K^k$ molecules suggests that they may be taking an alternate route to the cell surface, perhaps passing through endosomal compartments. The slightly slower maturation of these molecules also suggests a difference in intracellular trafficking between wild-type and mutant $K^b$ molecules.

Immature dendritic cells are known to contain high levels of class II molecules in MIIC compartments and low levels of class II on the cell surface. Upon acquisition of foreign antigen, these class II molecules are dissociated from the $\mathrm{Ii}$ chain and associated CLIP fragment so that they can bind to peptides generated from the exogenously-acquired antigens and be transported to the cell surface. This acquisition of antigen (in addition to other signals) initiates maturation of DCs, migration to lymph nodes, and expression of high levels of MHC class I, class II, and many other co-stimulatory and adhesion molecules important for T cell priming. The unpublished observation that there are high levels of class I molecules in the MIICs (personal communication, Ralph Steinman) of immature DCs strongly suggests that at least a subset of class I molecules are initially routed to MIICs, where they remain until they acquire peptide antigens. Maturation of dendritic cells presumably provides signals that result in expression of these class I complexes at the DC cell surface, an idea which is supported by the high levels of class I observed in mature compared to immature DCs.
Since class II molecules are dependent on endocytic motifs for their trafficking through MIICs, it is certainly feasible that any access to these same compartments by class I molecules is also dependent on similar cytoplasmic determinants. Two alternative models are presented for class I trafficking in APCs that may explain the CTL data presented in this thesis. One model is based on a proposed specialized recycling pathway present only in professional APCs and distinct from that of activated T cells. Under this model, certain class I molecules in APCs are preferentially internalized from the cell surface, recycled through endosomal compartments where peptide antigens are acquired, and re-expressed on the cell surface (see Figure 6-3). The second model proposes that certain class I molecules preferentially pass through endosomal compartments en route to the cell surface, where cytoplasmic mutants present peptide antigens for an unusually extended period of time due to their lack of internalization (see Figure 6-4). In this model, internalization and recycling of class I in activated T cells and APCs is governed by similar mechanisms. Thus, wild-type class I molecules (but not mutants) could potentially access endosomal compartments from the cell surface.
Figure 6-3: MHC class I trafficking model.

(next page) (A) In activated T cells, only wild-type K$^b$ molecules can be internalized and recycled via clathrin-coated vesicles (CCVs) efficiently, whereas none of the four K$^b$ cytoplasmic mutants can. (B) In APCs such as dendritic cells, CCV trafficking components recognize different cytoplasmic determinants, such that ΔSTSS and ΔVII mutants are internalized and recycled through endosomes preferentially. Wild-type K$^b$ molecules internalize and traffic through endosomes efficiently, ΔYSTSS molecules are considerably less efficient, and ΔY molecules are not internalized and recycled at all. Recycling through endosomal compartments in these cells presumably gives the class I molecules access to exogenously-derived antigens for cell surface presentation.
Figure 6-4: Proposed model of class I trafficking in APCs.

(next page) $K^b$ wild-type and $K^b$ mutant molecules are differentially sorted into CCVs from the trans-Golgi network (TGN). $K^b$WT and $\Delta Y$ molecules are shunted into the default secretory pathway, whereas $\Delta VII$ and $\Delta STSS$ mutants are sorted through endosomal compartments, where they may acquire exogenously-derived peptide antigens. $\Delta YSTSS$ molecules mostly traffic through the secretory pathway but there is some limited traffic through endosomes. From the cell surface, only $K^b$WT molecules may be efficiently internalized by CCVs and gain access to MIIC/endosomal compartments.
Before concluding this section, it would be useful to briefly discuss human MHC class I molecules, in particular HLA-C. This gene is one of the only examples of class I molecules that have been examined across several different species which lacks the highly conserved cytoplasmic tyrosine residue. In the HLA-C gene, the normal tyrosine codon TAC has undergone a point mutation to TGC, resulting in a cysteine residue in place of the tyrosine. This mutation was recently shown to be responsible for the HIV-nef protein’s inability to downregulate cell surface expression of HLA-C. Since HIV-nef normally downregulates expression of HLA-A and HLA-B molecules by increasing their accumulation into CCVs and endosomal compartments, the result supports the notion that the tyrosine residue is critical for MHC class I trafficking through these compartments.

HLA-C shows significantly less polymorphism and is expressed at significantly lower cell surface levels than either HLA-A or HLA-B. Several cases of allo-CTL directed against HLA-C have been reported in the literature. However, very few HLA-C-specific peptide-based CTL responses have been reported or characterized. It is therefore possible that the lack of a tyrosine residue in HLA-C causes an inability of these molecules to acquire exogenous antigens in APCs and effectively prime CD8+ T cells. Since our data shows that the cytoplasmic tyrosine is essential for VSV CTL responses in mice, it is feasible that some potential human CTL responses are abrogated in a similar manner.
Interestingly, the few HLA-C peptide-specific CTL that have been characterized recognize HIV-specific epitopes. DCs are thought to constitute a major repository of HIV and a major source of CD4+ T cell viral exposure. It is therefore quite possible that these HIV epitopes are being presented in DCs as endogenous antigens by HLA-C. Our results in fibroblasts, showing that ΔY mutations slow but do not prevent presentation of endogenously-synthesized viral antigens, support this hypothesis. The implications of this idea are not trivial, because it would imply that humans have only four, rather than the six class I alleles shared by other mammals, that are capable of generating optimal specific CTL responses in vivo.

The evidence of a drastically altered immune response to VSV in ΔY-expressing transgenic mice is an observation that may turn out to be useful for other aspects of human medicine. Since murine splenocytes have been reported to be refractory to VSV infection, it is difficult to explain away the observed splenic infiltration by inflammatory neutrophils as being an innate immune response to uncontrolled viral replication within the spleen. While much about this phenomenon still remains to be characterized, it would be interesting to explore whether any hereditary human diseases characterized by neutrophilia or splenomegaly are associated with cytoplasmic tyrosine codon mutations to HLA-A or HLA-B. Several HLA linkages to human diseases have been made or proposed. However, these allelic MHC linkages are based solely on extracellular serological determinants, meaning that none have taken the cytoplasmic domain into account.


6.3 Future Directions

Based on the results presented in this thesis, there are several avenues of research that are worthy of future investigation. The phenotypic analysis of the transgenic mice described by this thesis is still in its relative infancy, such that many questions about the cytoplasmic domain's \textit{in vivo} role remain unanswered. However, the data presented here has given rise to new questions, uncovered potentially novel regulatory mechanisms, and provided clues about what future studies may yield worthwhile information.

It is clear that more work needs to be done before we can assign a general role for the MHC class I cytoplasmic domain in the generation of \textit{in vivo} immune responses. CTL responses against other types of \(K^b\)-restricted epitopes, in particular those derived from Sendai virus (active and inactivated) and ovalbumin, need to be examined in the transgenic mice. If defects are found in the generation of CTL responses towards these antigens, it will provide clear evidence of a general role of the cytoplasmic tail in antigen presentation of both endogenous and exogenous antigens.

Failure to make a CTL response implicates a possible dendritic cell (or other APC) antigen presentation failure. To determine this, dendritic cells derived from all of the transgenic mice need to be analyzed \textit{in vitro} and \textit{in vivo} for class I surface expression, intracellular trafficking, and antigen presentation function of class I. There are several methods that could effectively test this hypothesis. In the first method, cultured dendritic cells are exposed to exogenous whole ovalbumin protein for various times. The appearance of \(K^b\)-OVA complexes on the cell surface can be monitored in two ways:
FACS staining with the K\textsuperscript{b}-OVA complex-specific mAb 25.D1 (described in Chapter 3) or stimulation of B3Z, a T cell hybridoma which expresses a K\textsuperscript{b}-OVA complex-specific TcR and expresses a lacZ gene with the IL-2 gene promotor in response to recognition. If ΔY DCs cannot present the processed ovalbumin peptide SIINFEKL in the context of K\textsuperscript{b} molecules, it will provide evidence of a DC failure in priming CTL responses \textit{in vivo}.

To test this directly, DCs cultured with ovalbumin (or inactivated Sendai virus) could be injected into naïve mice in order to measure the resulting OVA-specific (or Sendai virus-specific) CTL responses. If antigen-loaded DCs from a K\textsuperscript{b}WT-expressing animal can effectively prime CTL responses in ΔY mice, but DCs from a ΔY-expressing mouse cannot effectively prime CTL responses in K\textsuperscript{b}WT mice, it would provide direct evidence of a DC failure.

Any failure of APCs to present antigens could be examined further by immunofluorescence. In particular, K\textsuperscript{b}- and K\textsuperscript{k}-specific fluorescein-conjugated mAbs could be used to better define the subcellular compartments through which Class I molecules traffic in DCs and whether there is a difference in the subcellular localizations of the K\textsuperscript{b} cytoplasmic mutants. The K\textsuperscript{b}-OVA-specific mAb 25.D1 would also be very useful in this regard, because it could be used to visualize where OVA peptide-loading takes place, and whether any defect in the K\textsuperscript{b} cytoplasmic mutants is in trafficking, peptide-loading, or both. Co-localization studies using markers of known subcellular compartments, as well as sub-cellular fractionation methods could also be very useful to define allelic Class I localization in DCs.
Another avenue of research is to examine a different interpretation of the transgenic mouse CTL results. If the defect in CTL generation occurred at the level of the T cell and not at the level of the APC, it would imply that the Class I cytoplasmic domain had a role in T cell selection or activation. Crossing the transgenic animals to mice that are transgenic for a TcR that is K\textsuperscript{b}-restricted would probably provide answers about a potential role in T cell selection. Any differences in the resulting peripheral T cell repertoire or specificity between K\textsuperscript{b}WT-expressing and K\textsuperscript{b}-mutant expressing mice would provide evidence for this hypothesis.

To examine a potential role of the cytoplasmic tail in T cell activation, it must first be determined if antigen-loaded K\textsuperscript{b}WT-expressing DCs are capable of priming CTLs in ΔY mice. Failure to do so may indicate that the Class I cytoplasmic tyrosine is important for activation of CD8\textsuperscript{+} T cells. Since cross-linking of Class I molecules has been shown to induce signals in T cells, any Class I cross-linking that may occur during priming by APCs may provide a critical co-stimulatory activation signal.

The transgenic mice may also provide a means by which to test the receptor modulation theory of MHC Class I recognition by natural killer (NK) cells. NK cells are known to express killer inhibitory receptors (KIRs) that recognize and monitor the expression of self Class I molecules on the surface of most cell types. Binding and recognition of Class I by KIRs causes a signal to be generated in NK cells that inhibits target cell killing. Lack of Class I recognition causes the NK default killing program to be employed, and
the target cell is lysed. NK cells are thought to be very important in recognizing and killing potentially cancerous cells that often lose Class I expression as they undergo transformation to malignancy.

The receptor modulation theory postulates that NK cells adapt their surface expression levels of KIRs to the levels of Class I they encounter on the surface of cells within their environment. This presumably allows for fine-tuning of the inhibition signal intensity, such that there is thought to be an inverse relationship between the expression levels of Class I and NK surface expression of KIRs. The high levels of surface Class I that were observed in the K\textsuperscript{b} mutant-expressing transgenic mice provide a unique system in which to test the receptor modulation theory. If the theory is correct, K\textsuperscript{b}-specific KIRs (e.g. Ly-49C) on the surface of NK cells derived from K\textsuperscript{b} mutant transgenic mice should be expressed at significantly lower levels than in NK cells derived from K\textsuperscript{b}WT-expressing transgenic mice. All of the transgenic mice should express lower levels of KIRs compared to C57/BL control mice, which in turn should express less than C57/BL \times C3H F\textsubscript{1} mice.

The functional implications of the NK receptor modulation theory could also be tested using these transgenic mice. Interstrain NK killing assays should show a hierarchy of killing efficiencies, based on MHC Class I expression: NK cells isolated from the highest-expressing K\textsuperscript{b} transgenics should be able to kill target cells from K\textsuperscript{b}WT transgenics, C57/BL, and C57/BL \times C3H F\textsubscript{1} mice, which all express lower levels of K\textsuperscript{b}. Conversely, NK cells from C57/BL mice should not be capable of killing target cells
from any of the K\textsuperscript{b} transgenic mice but should be able to kill C57/BL x C3H F\textsubscript{1}-derived or K\textsuperscript{b}-negative target cells.

The study of MHC class I cytoplasmic mutants has implicated the existence of a fundamental mechanism that is responsible for controlling levels of surface expression in wild-type class I molecules. Understanding the mechanisms which control class I cell surface expression may have a direct clinical impact in disease therapy. For example, many tumours have lost the ability to express class I molecules, leading to their inability to be recognized and killed by potentially protective CTL. Organ transplants are still most often rejected due to alloreactions mounted against the donor tissue’s endogenous MHC class I. The ability to manipulate class I expression in these situations could be very useful. If phosphorylation of a cytoplasmic serine residue controls recognition of a class I cytoplasmic endocytic motif, then an obvious therapeutic target is the kinase or phosphatase that phosphorylates class I molecules. Alternatively, adaptins or other CCV components could potentially be modified to increase recognition and internalization of class I. Control of cell surface expression could also be achieved by modulating the amount of exon VII-deleted class I mRNA produced via alternative splicing, although the mechanisms underlying all of these processes remain obscure.
In conclusion, it is clear that there are several directions this project could take in the coming years. For example: studies to compare peptide repertoires bound by K^bWT and K^b mutant molecules; anti-tumor responses in the transgenic mice; and susceptibility of these mice to autoimmunity. These ideas were not discussed in detail, but are all worthy of future investigation. In this context, it is hoped that the biological systems generated in the course of this thesis work will continue to make contributions to our ultimate understanding of the true function of the Class I cytoplasmic domain.
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