Degree PHD Year 2006 Name of Author FLUTTER Barry Richard

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Investigation of Antigen Processing Using Partially Assembled MHC Class I Molecules

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Thesis submitted in part fulfillment for the examination of Doctor of Philosophy (PhD)

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Abstract

The processing of antigens for presentation to CD8+ T-cells in association with MHC class I molecules involves many chaperones and accessory proteins in the ER. Once processed into a trimeric complex of beta-2-microglobulin (β_2m), heavy chain and antigenic peptide the MHC class I molecule is complete and can traffic to the cell surface where it interacts with the T cell receptor of CD8+ T-cells.

The main focus of this work is the use of MHC class I fusion proteins, which by means of a covalent linker form partially assembled class I molecules. Using a fusion protein in which an antigenic HLA-A2 binding peptide is linked to β_2m (PB), it has been possible to show by immunoprecipitation that class I molecules can continue to interact with the peptide-loading complex after high affinity peptide has bound. This data is further supported by peptide release assays in which class I molecules are not released from the peptide-loading complex upon provision of high affinity peptide.

The expression of fusion proteins in which β_2m is linked to the heavy chain alleles HLA-B44 (β_2m-B44) or HLA-A2 (β_2m-A2), has highlighted an allelic difference in the requirement of MHC class I molecules for chaperones in the antigen processing pathway. Specifically it has been possible to show that while β_2m-A2 is able to express in murine fibroblast K41 cells, β_2m-B44 is not. This inability to express is not as a result of the absence of human tapasin, as previously reported, but because of some other unknown factor that is present in human cells but not in murine K41 cells. Interestingly the expression of β_2m-B44 in K41 cells also disrupts native MHC class I expression implying that the construct may be occupying chaperone molecules in the ER and delaying processing of mouse class I molecules. Furthermore, in the same system it has been possible to show that the major function of calreticulin is not the recruitment of β_2m to heavy chain, since the β_2m-A2 fusion protein, like normal class I expressed 8-10 fold less well in calreticulin deficient cells than in 'wild type' cells.

Acknowledgements

So many people to thank, so little space! I guess the best place to start is with my supervisors who have helped drive the project - and me at times. Bin for his scientific ideas and particularly Lucy for her moral support and scientific rigour. I must also mention how much I have valued Lucy's help in the last few months with corrections, references and assurances that I have a bright future whatever obstacles have seemed to hinder my path. For reagents I must also thank Jennifer Cox, Xiaoning Xu and of course Bin.

Staying in the workplace there are many more people who I need to thank for teaching me the skills and techniques and for their friendships that have been a valuable part of my life in London. Hemlata, Alka and Daniel for their help when I arrived and particularly Katy who I have been lucky enough to know would always be there if I needed help, support or a drunken night to forget about things when they weren't going right. Since she was a year ahead of me I also had the opportunity to gain from Katy's experiences along the adventure of a PhD. Hong Mei also deserves a special mention for being my closest research buddy. When she arrived a year into my course I realised how valuable it was to have somebody else in the lab using similar techniques and working in the same area – many times we have helped each other in the lab and I am grateful for her smiling face and her interest and help in my work in all times good and bad. I also want to mention Rachel, Marriane, Delali, Matt, Charles, Emma, Ding Jie, Parjeet and Mona who have all helped make the ICH a great place to work and have each helped me in some way over the last 4 years. In China thanks go to Fuliang and Zhou Juin for their friendship and to George Gao for having me.
I also need to mention the people I have lived with in London. Particularly Gill who was supportive in my decision to take up a PhD and whom I shared many happy times with and who always believed in me as a person and me as a scientist. Corrine and Gemma who shared in the good times at Campbell walk, and Preeti and Polly who still remain good friends and will for years to come. And more recently Piano and Petrushka for regular supportive Purrs and Licks.

For my inspiration I must thank some people from my past as well as those who have been around me for the last 4 years. John Knowland for training me how to think as a scientist, Robin for showing me how fun a lab can be... Tim Elliott for inspiring my

interest in the MHC and Immunology and to Cath, Angie, Iavor, Jules, Steve and Chris who were my support and life at Oxford.

The closer to my heart the people I write about get the harder it seems to be to express my thanks – and I have already failed in my objective to keep this down to 1 page! My family have and continue to be supportive and integral to my success in life. For many years they have nurtured me, my ambitions and occasionally my bank balance to keep me on a path, which so far I am glad to call successful. Alistair has been all I could have ever asked from a brother and he and my Dad have helped me to retain my philosophical approach to life. My dad also having done a PhD reassured me that I was on an achievable mission. My mum has often given me the emotional support I needed in my life and for that and for her confidence in me I cannot say enough.
Finally, to Rebecca I extend my love and thanks. You have kept my chin in the air many times and our home together has become a refuge in which we grow together.

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Abbreviations

β_2m	β-2 microglobulin
β_2m-A2	β_2m linked HLA-A2 construct (BA)
β_2m-B44	β_2m linked HLA-B44 construct (BB)
βgal	β- galactosidase protein
A	Adenosine
AIDS	Acquired Immune Deficiency Syndrome
APC	Antigen presenting cell
ATP	Adenosine tri phosphate
BA	β_2m linked HLA-A2 construct (β_2m-A2)
BB	β_2m linked HLA-B44 construct (β_2m-B44)
BCIP/NBT	5-bromo-4-chloro-3-indolyphosphate and nitroblue tetrazolium
bp	base pairs
C	Cytosine
CD	Cluster of differentiation
CMV	Cytomegalovirus
Cnx	calnexin
COPI	Coat protein I
COPII	Coat protein II
CRP	C-reactive protein
Crt	Calreticulin
CTL	Cytotoxic T Lymphocyte
DC	Dendritic cell
DMSO	Dimethyl sulphoxide
DNA	Deoxy-ribonucleic Acid
dNTP	Deoxy-nucleotide triphosphate
DRiPs	Defective ribosomal products
dsRNA	Double stranded RNA
eGFP	enhanced Green Fluorescent Protein
ELISpot	Enzyme linked immunospot assay
ER	Endoplasmic Reticulum
ERAAP	ER aminopeptidase associated with antigen processing
ERp57	Endoplasmic Reticulum protein 57 kDa
EtBr	Ethidium Bromide

FACS	Fluorescence activated cell sorting
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
g	Gravity
G	Guanine
GFP	Green Fluorescent Protein
GM-CSF	Granulocyte macrophage colony stimulating factor
HC	Heavy chain
HIV	Human Immunodeficiency Virus
HLA	Human Leukocyte Antigen
HRP	Horse radish peroxidase
IFNγ	Interferon gamma
Ig	Immunoglobulin
ILT	Immunoglobulin-like transcripts
IP	Immunoprecipitation
kDa	Kilodalton
KIR	Killer inhibitory receptor
Lac Z	Gene coding for β- galactosidase protein
LB	Luria Bertani
LPS	Lipopolysaccharide
MBL	Mannan binding lectin
MHC	Major Histocompatibility Complex
MW	Molecular weight
mRNA	Messenger RNA
NK	Natural killer cell
P/S/glu	Penicillin/ streptomycin/ glutamine
PB	peptide linked β_2m construct
PBA	Single chain construct of peptide, β_2m and HLA-A2
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline/ Tween
PCR	Polymerase Chain Reaction
PE	R-Phycoerythrin
Pfu	Plaque forming unit
PLC	Peptide-loading complex
PMA	Phorbol 12-Myristate 13-actetate

PTGS	Post Translational Gene Silencing
PVDF	Polyvinylidine diflouride
RISC	RNA induced silencing complex
RNA	Ribonucleic acid
RNAi	RNA interference
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
siRNA	Small interfering RNA
T	Thymidine
TAE	Tris/Acetate/EDTA
TAP	Transporter associated with antigen processing
TBE	Tris/borate/EDTA
TCR	T cell receptor
TdT	Terminal deoxynucleotidyl transferase
Th	T helper cell
TLR	Toll-like Receptor
TOPO	Topo-isomerase
TNF α	Tumour necrosis factor α
Tpn	Tapasin (TAP associated protein)
Treg	T regulatory cell
tRNA	Transfer RNA
U	International Units

1 Introduction

1.1 Immunity

1.1.1 The Immune System

The immune system is vital to the survival of complex organisms such as humans, but its job is highly complex and challenging. To function properly the immune system must effectively detect and eradicate 'foreign' bodies such as bacteria, viruses and mutated cells in the body, whilst tolerating healthy cells, food antigens and commensal organisms. However, since even the more complex 'adaptive' arm of the immune system has evolved alongside these various antigens for about 500 million years (Kasahara et al., 1995), most of the time it is able to distinguish correctly between harmful and harmless antigens and to act accordingly. Innate immunity, which has existed for even longer is common to all multicellular organisms studied (Beutler, 2004) and relies on the production of non variable molecules and receptors, which are able to recognise features common to a range of pathogens (Medzhitov and Janeway, Jr., 2002). Specific adaptive immunity, on the other hand, relies on the generation by somatic diversification of a huge and diverse repertoire of receptors, which can potentially recognise any foreign antigen (Burnet, 1959).

In order to infect and replicate in their host, microbes first have to pass the physical barrier of the epithelium. This in itself could be considered the first line of defense against infection, since the structure of epithelium with its tight junctions is designed to exclude pathogens. Beyond this physical barrier, the innate immune system possesses 'chemical weapons' including acids – fatty acids in the skin and hydrochloric acid in the stomach, enzymes such as lysozyme (Ogawa et al., 1971) and antimicrobial peptides such as defensins (Selsted et al., 1985). If these first lines of defense are breached then an immediate response to infection can be triggered by stimulation of pattern recognition receptors, which are able recognise motifs common to a range of pathogens. Pattern recognition receptors can be expressed on the cell surface, within cells or secreted into the bloodstream. Secreted proteins such as mannan binding lectin (MBL)(Super et al., 1989) and C-reactive protein (CRP)(Berman et al., 1986) are produced by the liver and bind to extracellular pathogens, typically resulting in stimulation of the complement pathway, leading to opsonisation and either lysis of the pathogen or engulfment by phagocytes. Cells of the innate immune system that express

pattern recognition receptors include macrophages, Dendritic Cells (DCs), mast cells, neutrophils, eosinophils and Natural Killer (NK) cells. Macrophages, dendritic cells and neutrophils are involved in the engulfment of bacteria and other opsonised material by phagocytosis. Through direct pattern recognition receptors, and more commonly via receptors for opsonins such as complement molecules, these phagocytes are important in the clearance of extracellular microbes. NK cells are involved in the recognition of infected cells: the mechanisms by which they do this will be discussed in further detail in section 1.1.5.

Ligation of pattern recognition molecules on cells of the innate immune system usually triggers them to initiate an inflammation response. This recruits and stimulates more cells of both the innate and adaptive immune systems. Stimulated cells of the innate immune system typically develop into short-lived effector cells whose chief aim is to clear the infection.

During the first few days of an infection while the innate immune system is attempting to clear or control the invading pathogen, the body also generates an 'adaptive' immune response. Adaptive responses are mediated by B cells, which produce antibodies that opsonise and lead to the destruction of extracellular foreign material, and by cytotoxic T cells, which are able to clear intracellular pathogens by killing the host cell. The adaptive response is triggered early during infection; usually in the draining lymph node whither antigen is transported from the site of infection by antigen presenting cells. However, on first encounter of a foreign antigen an adaptive response takes 5-7 days to develop. The chief reason for this delay lies in the very nature of the receptors involved. Because adaptive immunity uses such a wide range of receptors in order to be able to recognise any invading pathogen, the body cannot afford to produce each of these receptors at a high enough frequency to fight an infection. Instead, when the first encounter with antigen is made specific T and/or B cells proliferate, creating a pool of cells all carrying receptors specific for the pathogen. Only when sufficient numbers of antigen specific 'effector' cells have been produced can the adaptive response begin to fight the infection. However, once a response has developed the adaptive immune system is able to recognise pathogens through highly specific receptors and subsequently to form a memory compartment so that subsequent encounters with the same pathogen can be cleared more quickly.

The adaptive immune system relies on the innate immune system to alert it to infection and to direct its response. The key players in this process are thought to be dendritic cells (Nussenzweig and Steinman, 1980). Because dendritic cells are able to phagocytose pathogens and antigens, and to initiate either cytotoxic or humoral immunity in the lymph nodes by T cell stimulation, they are able to direct the immune response. Dendritic cells take up antigens at the site of an infection and in response to cytokines (primarily type I interferons), upregulate co-stimulatory proteins such as CD80 and CD86, increasing their potency as antigen presenting cells and driving stimulation of adaptive immune responses by presenting antigens to T cells.
Among the receptors of the innate immune system found on dendritic cells are the Toll-like receptors or TLRs. These were first discovered in *Drosophila* (Hashimoto et al., 1988) but soon after human equivalents were also found, which have been shown to be important in innate immunity (Anderson, 2000). To date 13 TLRs have been identified in mammals recognising a diverse range of microbial products such as lipids (TLRs 1,2 and 6), nucleic acids (TLRs 3, 7, 8 and 9) including double stranded RNA (dsRNA) and CpG DNA, and bacterial lipopolysaccharide (LPS) (TLR4) (Beutler et al., 2006). Stimulation of TLRs acts as a danger signal and often leads to inflammation and recruitment of other immune cells. When a dendritic cell is stimulated by TLR ligation, it may also influence the kind of adaptive response that it stimulates. Dendritic cells that recognise viral double stranded RNA (dsRNA) through TLR3 may be more likely to stimulate a response via IFNγ (Interferon γ), leading to cytotoxic T cell immunity. Whereas if TLR4 is ligated by bacterial LPS the production of an antibody mediated B cell response may be stimulated (Saint-Vis et al., 1998). By these mechanisms dendritic cells not only work in the innate immune system to destroy pathogens at the site of infection, but also direct responses of the adaptive immune response.

1.1.2 The Major Histocompatibility Complex

The Major Histocompatibility Complex (MHC) is a region of the genome, which is heavily involved in the function of the immune system. In humans it is a region located at chromosome 6p, of about 4Mbp (4 million base pairs) and is divided into three main regions: the class I region, found nearest to the telomere, followed by the class III and class II regions (The MHC Sequencing Consortium, 1999). The MHC plays a key role in disease resistance and susceptibility with linkage analysis showing that it has an influence on many autoimmune diseases including type 1 diabetes and rheumatoid

arthritis (Davies et al., 1994;Rotter and Landaw, 1984;Deighton et al., 1989), and some infectious diseases such as malaria and AIDS (Hill et al., 1991;Kaslow et al., 1996). In all there are about 60 genes within the MHC that code for proteins with immune functions, including most of the key genes necessary for antigen presentation. Antigen presentation by MHC class I and class II molecules is responsible for the stimulation of T cells, and is therefore central to the initiation of adaptive immune responses. The genes encoding MHC class I and class II molecules are highly polymorphic, in fact HLA-B (Human Leukocyte Antigen) is the most polymorphic locus so far identified in the human genome (Miretti et al., 2005). This variation is thought to have been driven by episodes of selection for resistance to infection, related to the ability of MHC alleles to present peptides derived from pathogens. Not surprisingly then, much of this variation is focused on the antigen binding groove of MHC molecules (van Bleek and Nathenson, 1991). Genes within the MHC region code not only for both class I and class II genes but also for vital genes in the antigen processing pathway of class I and class II molecules, including the Transporter associated with Antigen Processing (TAP) (Trowsdale et al., 1990;Deverson et al., 1990;Spies et al., 1990), tapasin (Herberg et al., 1998) and HLA-DM (Morris et al., 1994). Other genes in the region include complement proteins, TNF α (Tumour Necrosis Factor α) and within the extended MHC region genes coding for histones, tRNAs (transfer RNAs) and olfactory receptors. The high degree of polymorphism of the MHC region combined with strong linkage disequilibrium has stimulated much interest among immunologists and geneticists, since it indicates the evolutionary importance of genes within this region (Miretti et al., 2005;Traherne et al., 2006;Stenzel et al., 2004). Exactly how important antigen processing genes have been in driving linkage disequilibrium over evolution is not clear, since the presence of important housekeeping genes such as histones and tRNAs in adjacent regions may also have influenced the evolutionary process (Horton et al., 2004). Notwithstanding this, it is clear that the MHC region has been key to the evolution and functions of the adaptive immune system.

1.1.3 T cells

T cells are involved in the recognition of foreign antigens present in the body. The majority of antigens recognised by the T cell receptor (TCR) are in the form of peptide bound to a major histocompatibilty complex (MHC) molecule. T cells are generally classified in terms of their surface markers (or clusters of differentiation CD), and the

major subtypes of mature T cells are CD4+ and CD8+ T cells. Antigens can be presented to CD8+ T cells by MHC class I molecules, which are present on the surface of virtually all nucleated cells, and to CD4+ T cells by MHC class II molecules, which are found on the surface of professional antigen presenting cells, such as dendritic cells and B cells.

T cells precursors are made in the bone marrow and then migrate to the thymus, from which they take their name and where most of their early development occurs (MILLER, 1961;Cordier and Haumont, 1980). When T cells reach the thymus they are not yet committed to a particular lineage and are termed 'double negative' as they do not yet express CD4 or CD8 (Fowlkes et al., 1985); unlike T cells in the periphery, double negative T cells are also CD3 negative (Petrie et al., 1990). Key to the development of T cells in the thymus is the production of a functional T cell receptor (TCR) by the rearrangement of either the α and β chains, or the γ and δ chains of the TCR. In order to produce such a wide range of antigen specificities, TCRs are formed by the somatic rearrangement of the TCR gene loci. In the development of the $\alpha\beta$ T cell, which represents about 95% of the T cell output of the mature thymus, the β chain is the first to rearrange. The gene locus of the β chain codes for the variable regions made up of 3 segments known as V, D and J and a constant C region. The locus contains multiple different versions of each of the V, D and J segments and these can be combined in different combinations to create variation (Table 1.1)(Rowen et al., 1996). This variation is further increased by random nucleotide addition or deletion at each junction by the activity of TdT (Terminal deoxynucleotidyl Transferase). Although this leads to some out of frame rearrangements, the increase in diversity it creates is immense. Because of the arrangement of the TCR β locus and the presence of 2 copies of the gene locus, several possible attempts at rearrangement are possible increasing the chance of creating functional rearranged genes. If it is successfully rearranged, the β chain can then coexpress at the cell surface with the invariant pre-Tα chain and the CD3 complex. Expression of this pre-T cell receptor is essential for the survival of developing T cells (Fehling et al., 1995). T cells exhibit a 'double positive' (CD4+, CD8+) phenotype before rearrangement of the α chain (Mombaerts et al., 1992), which occurs in a similar fashion to β chain rearrangement, although the α chain contains no D segment. Once the TCR is fully rearranged and expressed, commitment of the T cell to a CD4+ or CD8+ lineage can be made according to the specificity of the TCR. T cells must be positively selected at this stage by interaction with MHC molecules on stromal

cells of the thymus and those T cells that are not positively selected, i.e. fail to interact with either class I or class II molecules expressed on the thymic epithelium, will die by apoptosis (Huesmann et al., 1991).

Conversely T cells expressing TCRs that weakly interact with either MHC class I or class II molecules in the thymus continue to develop. The interaction between the TCR and the MHC/peptide complex on its own is weak, so accessory receptors are also required to help in cell-cell adhesion. The most important co-receptors expressed on T cells are CD4 and CD8 proteins. Like the TCR these receptors interact with MHC molecules, but instead of binding to the antigenic regions, CD8 and CD4 bind to the non variable regions of MHC class I and class II molecules respectively. During thymic development those T cells that are reactive to MHC class II in cooperation with CD4, down regulate CD8 and *visa-versa* so that T cells that are released into the periphery express either CD4 or CD8 but not both (Kaye et al., 1989). The ε, δ, and γ chains of the CD3 complex are also vital for the expression and stability of the T cell receptor at the surface and the ζ chain is important for intracellular signalling upon recognition of antigens presented by MHC molecules. As well as undergoing positive selection via the TCR, T cells are also negatively selected in the thymus in a process known as central tolerance (Kappler et al., 1987). This negative selection involves the deletion of T cells whose TCR strongly recognises self-antigen in association with MHC molecules. This is thought to be a safety mechanism, which protects against autoimmunity.

Table 1.1. The number of variable gene segments encoding the Immunoglobulin and T cell receptor genes. *Adapted from* 'Immunobiology' *by Janeway, Travers and Walport.*

	Immunoglobulin genes		T cell receptor genes	
	Heavy chain	κ/λ light chains	α chain	β chain
Variable segments (V)	65	None	~70	None
Diversity segments (D)	70	6	52	13
Joining segments (J)	27	5/4	2	61

T cells leave the thymus in a naïve state and in order to become activated they require TCR recognition of an MHC-peptide complex in the presence of the appropriate co-stimulatory molecules (Liu and Janeway, Jr., 1992). This stimulation generally occurs in peripheral lymphoid organs such as draining lymph nodes and involves antigen

presentation by dendritic cells or other professional antigen presenting cells. Once activated T cells can proliferate and differentiate into a pool of effector and memory cells. Effector cells are strongly active cells, which persist during the infection but are gradually lost in the few weeks following clearance. Memory cells, as their name suggests, persist for much longer in the periphery although whether they can persist in the absence of antigen for the remainder of life is controversial (Kassiotis et al., 2003;Ciurea et al., 1999).

CD8+ Cytotoxic T lymphocytes (CTL) play an important role in recognition and elimination of cells expressing non-self proteins. MHC class I molecules are found on the surface of virtually all nucleated cells and each CD8+ CTL produced in the thymus carries a different TCR capable of recognising peptide antigen complexed with MHC class I. MHC class I molecules generally present intracellular antigens. On recognition of MHC class I carrying specific antigen, an activated CTL can kill the target cell, either by the release of granules containing perforin and other cytotoxic molecules, or via Fas-Fas ligand induced apoptosis. In this way CTLs can eradicate pathogen infected cells carrying foreign proteins and in some cases, transformed cells presenting mutated or overexpressed proteins.

CD4+ T cells do not directly kill pathogens or infected cells, but are required for effective B cell responses, and assist in the development of cytotoxic T cell responses. CD4+ T cells recognise peptide antigens associated with MHC class II and these antigens are generally derived from extracellular proteins. Only specialised cells such as dendritic cells, macrophages and B cells express MHC class II and this reflects the specialised role of CD4+ T cells in helping other branches of the immune system. CD4+ T 'helper' cells are generally categorised as being Th0, Th1 or Th2 (Mosmann and Coffman, 1989) (Kamogawa et al., 1993) although the point at which their lineage becomes committed to either Th1 or Th2 from the 'neutral' Th0 precursor remains unclear (Grogan and Locksley, 2002). Th1 cells are induced by cytokines such as Il-12 and IFN-γ and they produce IFN-γ and IL-2, which activate macrophages and assist in the development of a cytotoxic T cell response. Th1 cells also suppress the production of Il-4 and Il-10 by surrounding cells, cytokines that promote a Th2 response. Th2 responses produce IL-4, Il-5 and Il-10, which are important for the development of the humoral response, stimulating B cells into differentiation and class switching (Berton et al., 1989).

Dendritic cells are probably the major stimulators of naïve CD4+ T cells since they are able to present antigen along with powerful co-stimulation (Nussenzweig and Steinman, 1980). Dendritic cells that have picked up antigen at the site of an infection are likely to be particularly potent stimulators since they will have upregulated co-stimulatory molecules such as CD80 and CD86 as discussed in section 1.1.1. They may also influence CD4+ T cells into stimulation of either a Th1 response assisting CD8+ T cells, or a Th2 response assisting B cells. The requirement of CD4+ T cell help for the activation of naïve B cells and CD8+ T cells is important in directing the correct type of response. CD4+ T cell help also allows powerful stimulation of effector B and T cells when and only when harmful antigen is detected. The requirement of this second signal of stimulation for naïve effector cells also guards against autoimmunity. For instance, if a B cell has encountered and endocytosed bacteria or other material because of recognition of its B cell receptor it can present antigens derived from the endocytosed material on class II molecules (see 1.2.1). Activated CD4+ Th2 T cells can then recognise this antigen and stimulate the B cell to proliferate producing effector cells and to differentiate allowing optimisation of the antibody response.

Finally, a more recent finding and area of intense work in recent years are T regulatory (Treg) cells. Unlike most other T cells, Tregs typically do not respond to antigen by proliferation *in vitro*, and are therefore termed anergic. Their role appears to be in the suppression of the response of other T cells. Several different subtypes of Treg have now been identified but all are implicated in the production of peripheral tolerance. The most well characterised subset is the CD4+ CD25+ thymically generated Treg, which can both decrease the severity of autoimmunity (Asano et al., 1996;Sakaguchi et al., 1996) and play a role in controlling immune responses to pathogens (Suvas et al., 2003).

1.1.4 B cells

Unlike T cells, which migrate to the thymus from the bone marrow to undergo their early development, the production of immature B cells occurs in the bone marrow. During this process, somatic rearrangement of the immunoglobulin (Ig) gene loci takes place. The rearranged genes then code for a functional immunoglobulin receptor (IgM) made up of a 2 identical heavy chains and two identical light chains (either κ or λ)

(Edelman, 1970). The variable sections of both heavy and light chains contribute to the antigen specificity of the two identical antigen binding sites on the folded Ig molecule. Like the T cell receptor, the great variation in the specificity of different immunoglobulins is made possible by the great number of possible rearrangements of the gene loci (Tonegawa, 1983) (Table 1.1).

Development of B cells in the bone marrow occurs independently of antigen, so B cell tolerance relies on negative selection in the periphery. Immature B cells, which recognise antigen in the periphery are either eliminated or become anergic. Those that survive this selection procedure go on to become naïve B cells expressing both IgM and IgD. Different isotypes of immunoglobulin such as IgM and IgD have the same specificity but differ in their avidity and in their properties of opsonisation, complement activation and other interactions with the immune system owing to differing 'Fc' domains coded for in the C region of the Ig genes (Morgan and Weigle, 1987). In order to be stimulated to produce antibody, B cells must bind to antigen and receive stimulatory signals either from CD4+ T-helper cells (Claman and Chaperon, 1969) or by recognition of microbial constituents. T cell help can cause proliferation, differentiation, class switching and antibody production by B cells.

Antibodies are soluble forms of immunoglobulin, which can be secreted from B cells and eliminate disease in several ways. 1) They can neutralise pathogens which normally reside within cells but which must spend some of their life cycle outside cells in order to reproduce or move to other cells. 2) They can opsonise extracellular pathogens, leading to the recruitment of complement and either lysis of the pathogen or engulfment by phagocytic cells. 3) They can bind to and neutralise toxins. 4) They can release cytokines and recruit other cells of the immune system to the site of infection (Janeway et al., 1999).

Activated B cells do not necessarily only produce one specific antibody in their lifespan, but with CD4+ T cell help can undergo somatic hypermutation in germinal centres (French et al., 1989). During this process the DNA sequences coding for the variable loops of immunoglobulin are randomly mutated to modify their specificity. Although most mutations may result in loss of affinity, it can be seen from the development of antibody responses that high affinity antibodies are positively selected. In this way antibody responses increase in affinity during an infection or on multiple exposure to the same antigen. Because there is a B cell memory pool, cells producing

high affinity antibody are available to eliminate pathogens quickly and efficiently upon second encounter with antigen.

1.1.5 Natural Killer Cells

Natural Killer (NK) cells are able to recognise and kill a wide range of both mutated and infected cells. NK cells can eradicate cells either through antibody dependent cell cytotoxicity (ADCC) or through 'natural killer' activity. The exact signals and mechanisms upon which they carry out the second of these functions is not clear but it is thought that NK cell killing is controlled by a balance between activating and inhibitory signals received through its cell surface receptors. NK cells display a range of germline encoded receptors able to recognise proteins such as MHC class I on the surface of other cells. It is thought that NK cells can kill target cells which are not expressing class I molecules (Ljunggren and Karre, 1990). NK cells recruited to the site of an infection often become activated by cytokines such as IL-12 and Type I interferons. When activated, NK cells produce IFNγ, which is known to upregulate MHC class I on bystander cells. This may offer help to cytotoxic T cells, since they rely on recognition of antigen in association with class I molecules. But it may also allow easier detection of virally infected cells that are evading CD8+ T cell immunity through downregulation of surface MHC class I expression. Many viruses downregulate native protein synthesis in their host cells and may be detected and eliminated by NK cells early during infection. Other viruses specifically downreglate MHC class I in their host cell to evade cytotoxic T cell killing, however if these cells fail to respond to IFNγ and instead maintain low cell surface class I, then NK cells may be able to detect and kill these cells. The major class of receptors on NK cells are known as Killer Inhibitory receptors (KIRs) and although many of the proteins are based on fundamentally different protein folds in humans and mice, the proteins seem to fulfil very similar roles (Martin et al., 2002). It is interesting that like the MHC region the major coding regions for NK receptors are highly polymorphic and have also been shown to have some disease association, suggesting that they may have been important during evolution as well as for our current immunity (Tay et al., 1999;van der Slik et al., 2003).

1.2 Overview of Antigen Presentation

The role that antigen presentation plays is absolutely vital to the adaptive immune response and has been extensively reviewed (Immunological reviews. Vol 207;Villadangos et al., 2005;Flutter and Gao, 2004). Unlike B cells, which recognise a whole variety of antigen types including those on live bacteria, T cells usually recognise small processed peptide antigens presented to them by MHC molecules. The contrasting roles of CD4+ and CD8+ T cells are reflected in the origin of peptide antigens that are presented by MHC class II and class I molecules.

1.2.1 MHC class I and class II

MHC class I antigen presentation occurs in virtually all nucleated cells of the body and allows peptide antigens from a random sample of proteins expressed within the cell, to be seen at the cell surface by passing CD8+ T cells. This presentation of intracellular antigens allows T cells to detect infected or mutated cells and take action against them.

MHC class II antigen presentation occurs on only a specialised subset of cells known as professional antigen presenting cells. These are mainly dendritic cells, macrophages and B cells as well as epithelial cells of the thymus involved in the development of T cells. Class II molecules interact with CD4+ T cells, whose major job it is to stimulate responses by either CD8+ T cells or B cells that have come into contact with their specific antigen. It therefore makes sense that class II molecules present antigens derived from extracellular proteins that have been taken up into the cell by phagocytosis or endocytosis. Endocytosed material is broken down in the phago-lysosomal pathway and developing class II molecules are exported from the ER to intercept this pathway. Class II molecules are therefore able to load peptide antigens at the site where they are created. Peptide antigens displayed by class II molecules are generally 13-17 amino acids in length, although because they can overhang from the binding groove of class II molecules they are not constrained in length.

1.2.2 Cross-presentation

Given that the function of CD8+ T cells is to kill infected cells, it is logical that MHC class I molecules should present intracellular antigens to CD8+ T cells, and early *in vitro* experiments on class I presentation seemed to confirm this (Morrison et al., 1986).

However, this presents a problem in the initial activation of naïve cells, since if only intracellular antigens are presented on class I molecules, CD8+ T cell responses to a virus would have to be stimulated by cells infected with that virus. In contrast to professional APCs, such infected cells may not have the capacity to stimulate naïve T cells. Similarly, it did not fit with *in vivo* data that clearly showed that when exogenous antigen was administered, reactive CD8+ T cells could be generated which were specific for the given antigen, presented on MHC class I (Bevan, 1976). Subsequently it has been shown that presentation of exogenous antigen on MHC class I molecules, termed 'cross-presentation', occurs in a small subset of cells, principally macrophages and dendritic cells (Rock et al., 1993).

Dendritic cells are thought to be by far the most important cells in cross-presentation, and two different mechanisms have been identified by which it can occur. In the first, antigen is exported from the endosome into the cytosol and then enters the classical pathway for class I antigen presentation (see section 1.3) (Kovacsovics-Bankowski and Rock, 1995). In the second pathway, it is proposed that class I molecules might be loaded with antigen in the phago-lysosmal pathway rather like the loading of class II molecules (Pfeifer et al., 1993). Recently it has been shown that there is a 'donation' of membrane from the ER to the phagosome during phagocytosis, and that class I molecules and all the accessory proteins necessary for antigen processing are present in the 'donated' membrane (Gagnon et al., 2002;Ackerman et al., 2003). Class I molecules are therefore able pick up antigen in the phagocytic pathway. However, since the proteasome and the Transporter associated with Antigen Processing (TAP) (see section 1.3.3) seem to be important for cross presentation *in vivo*, it is hypothesised that in the majority of cases, antigen is still exported into the cytoplasm for degradation, before being imported back into the phagosome and associating with class I (Huang et al., 1996).

Some researchers still argue that although cross-presentation can be demonstrated *in vitro* or *in vivo* using artificial antigens heavily laden with adjuvants, it is not likely to be a process that is important in natural immunity against viruses (Zinkernagel, 2002). They propose that the requirement for dendritic cell stimulation in the response results from the importance of CD4+ T cell help and that CD8+ T cell stimulation does indeed involve antigen presentation by infected cells. Among the evidence supporting this

theory is the absence of cross priming shown in some allogeneic cell transfer experiments (Kundig et al., 1996).

1.3 MHC class I

The importance of MHC molecules was first noticed in 1974 when Zinkernagel and Doherty found that virus specific cytotoxic T lymphocytes only attacked infected cells bearing the same MHC class I molecules as the CTL itself (Zinkernagel and Doherty, 1974). This phenomenon is known as MHC restriction, and the implication behind the finding was that MHC class I molecules have a role in CTL recognition of infected cells. However, the mechanism behind MHC restriction remained unclear for some years. In 1982, Alain Townsend at Oxford University showed that CTL clones specific for influenza A proteins could respond to target cells expressing incomplete viral glycoproteins (Townsend and Skehel, 1982), and then showed that specific CTLs could kill non-infected cells simply by pulsing the cell with specific peptides from proteins of the flu virus (Townsend et al., 1986). From this it was deduced that the antigenic determinant of the virus was not full length protein, but short peptides derived from proteins expressed in the cell. These data, along with the concept of MHC restriction, suggested that MHC class I molecules play a role in CTL recognition by facilitating the presentation of peptide antigens.

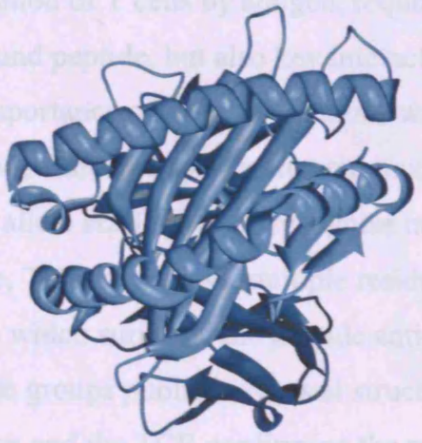
1.3.1 Class I structure

In 1987, Pamela Bjorkman and Don Wiley solved the crystal structure of the MHC class I complex and noticed an area of electron dense material in a groove formed by the MHC class I molecule (Bjorkman et al., 1987a). By refolding the class I complex with homogenous peptide they then confirmed that this electron dense region was bound peptide. Shortly after this, naturally processed viral peptides were isolated from infected cells by acid extraction and sequenced. It was shown that the peptides eluted from the cell depended upon the MHC class I alleles present (Rotzschke et al., 1990).

The highly polymorphic genes, which code for the MHC class I heavy chain are found at 3 loci within the MHC. Therefore each individual carries up to 6 different heavy chain alleles (three from each parent). In contrast, the light chain or β-2-microglobulin (β_2m) as it is more commonly known, is relatively non polymorphic and is invariant within the complex. The final component of the trimeric MHC class I complex expressed at the cell surface is the bound peptide; this is the antigenic element of the complex and can potentially be derived from any protein resident within the cell. The

crystal structure of the class I molecule has greatly enhanced our understanding of how peptides binds to class I and has helped to explain how each allele of class I can interact with many peptide antigens (Bjorkman et al., 1987a). There are 4 protein domains in the MHC class I heterodimer three of which, the α1, α2 and α3 domains are formed by the heavy chain while β_2m forms the 4th domain. The α1 and α2 domains share a very similar structure consisting of 4 antiparallel β-strands followed by one long α-helix. The two domains interact in such a way that the 8 β-strands form one large β-sheet crossed by two α-helices leaving a long groove down the centre. This long groove, bounded by the β-sheet at its base and α-helices at either side, forms the peptide binding site (Fig. 1.1).

Figure 1.1. Ribbon diagram showing the crystal structure of HLA-A2. *Top view of HLA-A2 showing the peptide groove, formed at its base by the β-sheet and at the sides by the α-helices of the α1 and α2 domains (cyan). α3 domain shown in blue.* *Adapted from* - Cellular and Molecular Immunology **1** No.1; *Flutter,B. Gao,B.* **MHC Class I Antigen Presentation - Recently Trimmed and Well Presented**



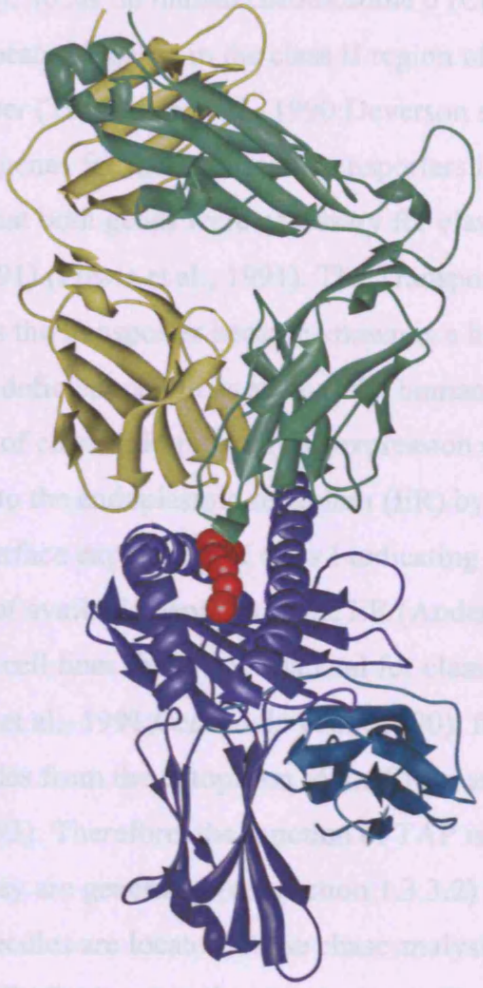
The bound peptide in the class I complex is generally 8-10 amino acids in length and forms hydrogen bonds with residues of the groove at both its N and C-termini. Other interactions at certain key anchor residues securely bind the peptide to the class I molecule (Falk et al., 1991). The anchor residues are determined by the shape of the peptide groove and vary from one heavy chain allele to another. Although binding to the heavy chain requires certain anchor points in the peptide sequence, often more than one amino acid can act as the anchor residue. There is much more flexibility in the other amino acids of the peptide meaning that each heavy chain allele can bind to a wide

range of peptide sequences. It is no surprise to find that the majority of the polymorphic residues of class I are found around this peptide groove allowing variation in the shape and electrostatic properties of the groove so that each heavy chain allele can bind a different array of peptides by means of different anchor residues. This maximises the number of peptide antigens which an individual can present as each of the six alleles carried will be able to bind and present a different repertoire of peptides (Bjorkman et al., 1987b).

1.3.2 TCR recognition of class I

TCR recognition of the peptide bound to the groove of the MHC class I molecule is the key to the specificity of the immune response made. Specific peptide sequences recognised by T cells are usually known as epitopes (although some researchers argue that this usage is not strictly accurate, since the term epitope originally referred to residues recognisable at the surface of a protein (Yewdell, 2005)). However, the stimulation of T cells by antigen, requires not only interactions between the TCR and the bound peptide, but also key interactions with residues of the class I molecule itself. The importance of these interactions was highlighted by Ajitkumar and co-workers, in 1988, who showed by site directed mutagenesis, that unlike antibodies to the mouse class I allele H2-K^b, which recognise randomly distributed residues on the class I surface, TCRs recognise multiple residues located exclusively on the surface of the α-helices which surround the peptide antigen (Ajitkumar et al., 1988). In 1996 two separate groups published crystal structures of the docking of an MHC class I peptide complex and the TCR confirming the multiple interactions between the TCR and both the antigenic peptide and the surrounding residues of the class I molecule (Garcia et al., 1996;Garboczi et al., 1996).

Figure 1.2. Ribbon diagram showing the crystal structure of a TCR docking with class I. *Crystal structure of HLA-A2 (heavy chain - dark blue, β_2m – cyan) bound with tax peptide (red) docking with antigen specific TCR (α chain – yellow, β chain – green).* *Adapted from* - Cellular and Molecular Immunology **1** No.1; *Flutter,B. Gao,B.* **MHC Class I Antigen Presentation - Recently Trimmed and Well Presented.**



1.3.3 Production and Provision of peptides for class I

1.3.3.1 MHC class I presentation failure

In 1986, Peter Cresswell's lab reported a mutant cell line 174XCEM.T2 (T2) in which MHC class I heavy chain and β_2m are expressed normally within the cell, but fail to form dimers that are processed and transported to the cell surface. They proposed that an additional molecule, absent from T2 cells, and encoded by an HLA-linked gene is

necessary for efficient assembly of class I (Salter and Cresswell, 1986). Cerundolo and Townsend demonstrated that .174, a parent cell line of 174XCEM.T2 has lost a function required for presentation of intracellular viral antigens with class I molecules of the major histocompatibility complex (MHC), but retains the capacity to present defined epitopes as extracellular peptides. They mapped the genetic defect of .174 cells to a position within the MHC locus on human chromosome 6 (Cerundolo et al., 1990) and 3 separate groups soon located 2 genes in the class II region of the MHC encoding a putative ABC transporter (Trowsdale et al., 1990;Deverson et al., 1990;Spies et al., 1990). By transfecting genes for these putative transporters into deficient cell lines it was possible to show that both genes were necessary for class I antigen presentation (Spies and DeMars, 1991) (Powis et al., 1991). The Transporter associated with Antigen Presentation or TAP, as the transporter became known is a heterodimeric protein of TAP1 and TAP2. TAP deficient cell lines such as the human B cell line T2, display low cell surface expression of class I, despite normal expression of both heavy chain and β_2m. Peptides targeted to the endoplasmic reticulum (ER) by means of a signal sequence restore cell surface expression of class I indicating that the mutant cell suffers because of an absence of available peptides in the ER (Anderson et al., 1991). After it was shown in deficient cell lines that TAP was vital for class I presentation (Salter and Cresswell, 1986;Powis et al., 1991;Cerundolo et al., 1990), further study revealed that TAP translocates peptides from the cytoplasm to the ER in an ATP dependent fashion (Androlewicz et al., 1993). Therefore, the function of TAP is to transport peptides from the cytoplasm where they are generated (see section 1.3.3.2) to the ER where newly synthesised class I molecules are located. Pulse chase analysis showed that without provision of peptide by TAP, class I molecules do not traffic to the Golgi body but instead are slowly degraded in the ER (Knittler et al., 1999).

1.3.3.2 Generation of peptides

Peptides for class I loading are produced from proteins in the cytoplasm, which are constitutively degraded by the proteasome at variable rates. The proteasome generally breaks down proteins of the cell that have been marked for degradation by ubiquitination (Kloetzel, 2004). This is a constitutive process, which allows the turnover of cellular proteins. However, in some cases proteins may be specifically tagged for degradation by the proteasome at an increased rate. It has been hypothesised that the majority of antigens presented by class I molecules may actually be derived from

defective ribosomal products (DRiPs) (Reits et al., 2000). In other words, they may arise from newly synthesised non-functional proteins, and this is supported by the fact that inhibition of protein synthesis seems to affect class I presentation (Khan et al., 2001). However, DRiPs cannot be the only source of antigens for class I, since peptides can also be presented from whole proteins introduced into permeabalised cells. Furthermore, cross-presentation cannot be carried out by presentation of DRiP derived antigens, since by definition cross-presented antigens are derived from exogenous material.

The degradation products of proteins are dependent on the components present in the proteasome involved in protein cleavage. Stimulation by the cytokine IFNγ alters the components of the 26S proteasome by incorporating the subunits LMP2, LMP7 and MECL-1 and producing an "immunoproteasome" (Gaczynska et al., 1993). This immunoproteasome tends to produce longer peptides and cleaves after hydrophobic and basic residues, which are transported more easily by TAP and are more suitable for binding to MHC class I. In this way the carboxyl terminus of peptides which bind to class I is produced directly by the proteasome. In contrast the proteasome does not seem to cut peptides exactly to size at their amino terminus. Since peptide epitopes vary, this presumably allows the proteasome to produce a wide range of peptide precursors, while it would be much more difficult to produce the exact peptide for class I binding. The dominant epitope of wild type ovalbumin presented by K^b is SIINFEKL, and both ovalbumin and SIINFEKL are commonly used as model antigens for class I presentation. Using a modified ovalbumin molecule, the relative quantities of the related peptide epitope SIINFEHL and its amino-extended precursors have been analysed (Paz et al., 1999). It was shown that although the peptide that is loaded onto class I and presented to T cells is the 8-mer SIINFEHL the levels of this peptide within the cell are very low. The 9-mer KSIINFEHL is present at significantly higher levels but localises only to the ER and its production is dependant on functional TAP. The 10-mer and 11-mer precursors of the peptide on the other hand are found at higher levels still and mainly reside in the cytoplasm. These findings suggest that the proteasome produces amino-extended versions of the peptide, which are subsequently trimmed at their amino terminus to produce peptides for class I binding.

1.3.3.3 ER Aminopeptidase associated with Antigen Processing

Recent work has shown that the main enzyme involved in peptide trimming is located within the ER (Serwold et al., 2002;Saric et al., 2002;York et al., 2002). This enzyme, the ER Aminopeptidase associated with Antigen Processing (ERAAP or ERAP1), was isolated from mouse spleen cells by ion exchange chromatography and its activity was assayed using leucine *p*-nitroanilide (Serwold et al., 2002). The isolated protein had a molecular mass of roughly 100 kDa and its aminopeptidase activity was inhibited by leucinethiol. By trypsin digestion and mass spectrometry the peptide fingerprint was determined and used to search the National Centre for Biotechnology Information database of proteins. The matched protein was a murine leucine aminopeptidase. Serwold et al. went on to show that this protein was Endo H sensitive, colocalised with the ER proteins BiP and gp96, and that the level of protein in different tissue types correlated well with MHC class I expression. Not only this but like heavy chain, tapasin, TAP and other components of the class I pathway, expression of ERAAP was also increased 10-fold in fibroblasts when cultured with IFNγ. Concomitant with this increase in expression is an increase in aminopeptidase activity (Saric et al., 2002). Assays on the activity of ERAAP show that it cleaves peptides of $\geq$ 10 amino acids at a high rate, cleaves 9 amino acid peptides at a reduced rate and has little or no activity for peptides of $\leq$ 8 amino acids (Saric et al., 2002). This range of cleavage specificity ought to be ideal for the production of the 8-9 amino acid peptides, which form the majority of epitopes presented by class I molecules.

Using RNA interference (RNAi) two groups have recently succeeded in suppressing the activity of ERAAP (York et al., 2002;Serwold et al., 2002). This work has confirmed that ERAAP is important in producing the peptide repertoire for class I presentation. Interestingly, although in some cases suppression of ERAAP correlates with the loss of peptide presentation and specific CTL response, certain epitopes appear to be produced and presented more efficiently in the absence of ERAAP (Serwold et al., 2002). This may reflect the fact that some peptides are produced independently of ERAAP and they may face less competition for class I binding when ERAAP is suppressed and consequently present better.

York et al. noticed that surface expression of K^b in HeLa cells was actually increased when ERAAP was suppressed using RNAi. They also showed by pulse chase experiments that the rate of assembly of HLA-A, B and C was increased under RNAi to

ERAAP. They attributed this to the destruction of peptides by ERAAP that bind to class I molecules. In this situation provision of peptide is presumably limiting the rate of class I trafficking and hence, loss of any peptides which can bind to class I will decrease surface expression. In fact a low level of aminopeptidase activity either from the remaining ERAAP or from other aminopeptidases, might be ideal as it will slowly trim precursors that require processing without destroying epitopes which already bind to class I. Conversely when cells are stimulated with IFNγ, although the proteasome seems to produce little or no more peptides of the correct length for binding, it does produce 2-4 fold higher rates of amino-extended versions of the peptides (Cascio et al., 2001). Peptides now cease to be limiting and may frequently require trimming before being loaded onto class I. ERAAP will trim peptides that are imported by TAP and produce the correct epitopes to bind to class I. Although some epitopes may be destroyed and presented at a lower level (Serwold et al., 2002) the overall amount of surface class I is now as high or higher than when ERAAP is suppressed (York et al., 2002). Crucially, the range of peptides presented may also be increased which is an important factor in development of immune response during infection and inflammation, when IFNγ is high.

Paz et al. (Paz et al., 1999) found that the trimming of the K^b binding peptide precursor KSIINFEHL was strongly increased in the presence of K^b, but not with the irrelevant K^d. Along with evidence from Saric et al (Saric et al., 2002) that ERAAP is responsible for trimming amino-extended versions of the very similar SIINFEKL peptides, we are led to the intriguing possibility that ERAAP may cleave peptides which are already in association with class I. This suggests that ERAAP may be recruited to the loading complex in order to trim peptides with a high affinity for the specific class I alleles present in the complex. However, TAP immunoprecipitates do not seem to include ERAAP suggesting that any interaction it has with the peptide-loading complex is weak and transient (Ackerman et al., 2003).

1.3.4 MHC class I trimeric complex

As shown from extensive work on class I antigen processing, including the crystal structure showing the docking of class I with the TCR (Garcia et al., 1996;Garboczi et al., 1996), recognition of antigen by T cells requires a trimeric class I complex of β_2m, heavy chain and peptide. The folding of this complex occurs in the endoplasmic reticulum (ER) and involves three crucial stages: synthesis and folding of the heavy chain and β_2m, provision of peptides for class I loading, and the loading onto heavy chain of peptide with high affinity for the peptide groove[1]. Only once these processes have occurred, can loaded class I molecules be exported to the cell surface for surveillance by T cells (Fig. 1.3).

1.3.4.1 Folding of the peptide receptive β_2m-HC heterodimer

Shortly after or during synthesis in the ER, heavy chain is bound by the molecular chaperone calnexin (cx) (Degen and Williams, 1991;David et al., 1993), which may act to stabilise and protect it from degradation (Williams, 1995) or facilitate folding and association with β_2m. Calnexin is a membrane bound molecular chaperone containing a lectin site which recognises monoglucosylated N-oligosaccharides (Hammond et al., 1994). Human class I molecules contain one N-glycan site whilst mouse class I molecules have two or three such sites, and this may explain differences in the observed binding of calnexin to developing class I molecules. Thus, in human cells calnexin is predominantly found associated with free heavy chain, and upon binding of β_2m to heavy chain, calnexin is dropped in favour of another similar chaperone calreticulin (crt) (Sadasivan et al., 1996). In contrast, in mouse cells the interaction with calnexin can persist beyond β_2m binding and possibly right up until class I export from the ER (Degen and Williams, 1991). Although most heavy chain molecules appear to associate with calnexin transiently in human cells and for longer in mouse cells, it is important to note that in both human and mouse cells with calnexin deficiency, class I peptide loading and cell surface expression are normal (Scott and Dawson, 1995) (Sadasivan et al., 1995). This leads to the conclusion that calnexin is dispensable in class I processing, possibly owing to some ER chaperone redundancy through calreticulin or BiP. What does seem to be clear is that only once the heavy chain has been assembled into a dimer

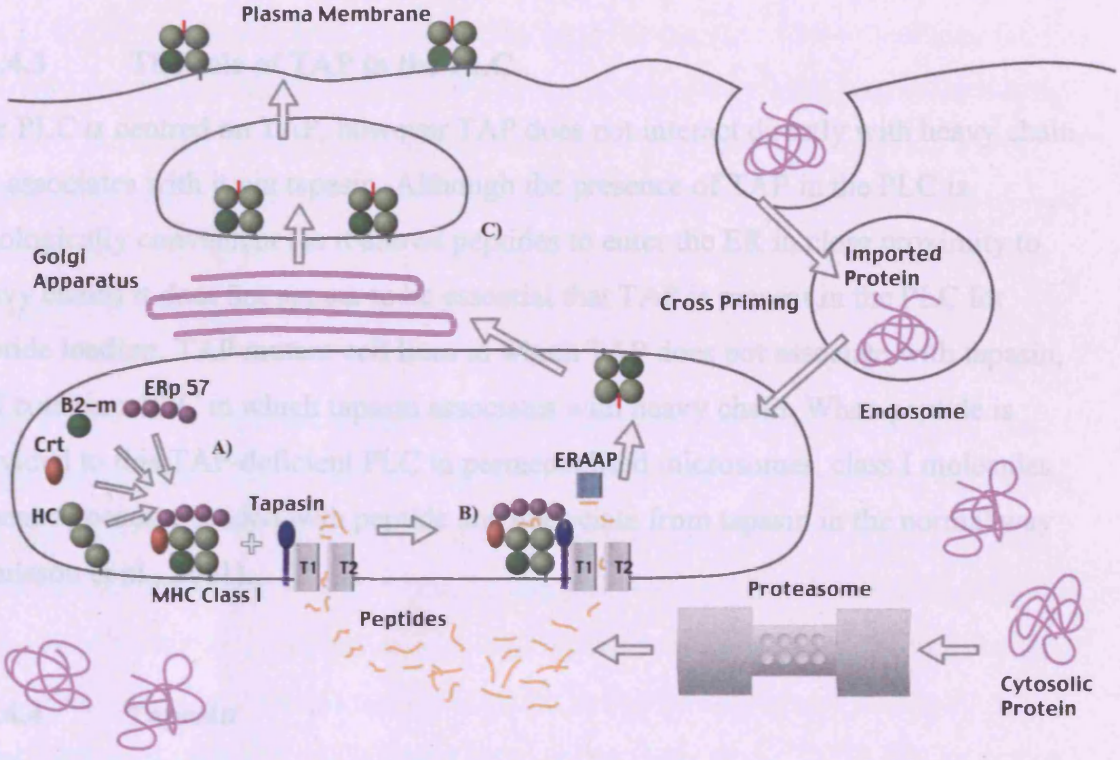
[1] Here and in other places in this thesis, the term 'high affinity' refers to the binding affinity of the peptide for the binding groove of the MHC class I molecule and is not related to the affinity of the interaction between the T cell receptor and the MHC/peptide complex.

with β_2m can it bind to calreticulin (Fig. 1.3B) and progress to the peptide-loading complex (PLC) (Farmery et al., 2000).

1.3.4.2 The peptide-loading complex

Interestingly not only is TAP necessary for the provision of peptides to class I molecules, but it also immunoprecipitates with both class I heavy chain and β_2m (Androlewicz et al., 1994). *In vitro* this interaction is lost upon binding of peptide to class I suggesting that the β_2m-HC heterodimer is in complex with TAP before it is loaded with peptide, but that on peptide binding it is released to traffic to the cell surface (Suh et al., 1994). More detailed immunoprecipitation work has shown that at least three other proteins are also bound to TAP and class I prior to peptide loading. Together these proteins form what is known as the peptide-loading complex (PLC). The PLC is centred on the TAP1-TAP2 dimer and contains the β_2m-HC class I dimer and tapasin (tpn) at a stoichiometry of 1 TAP: 4 tapasin: 4 heavy chain (Ortmann et al., 1997). As well as this other components including the chaperones calreticulin, calnexin (in murine cells) and the protein disulfide isomerase ERp57 (Endoplasmic Reticulum protein 57) are also found in the complex (Fig. 1.3C).

Figure 1.3. Class I antigen presentation pathway.
A) Heavy chain (HC) disulfide bridges form and it associates with β_2m (B2-m) allowing calreticulin (Crt) to bind. B) The class I molecule enters the mature complex and binds to peptides provided by TAP (T1 and T2). Tapasin is found disulfide bonded to ERp57. C) Once loaded with peptide, class I is released from the loading complex and traffics to the cell surface via the Golgi network. ERAAP - ER Aminopeptidase associated with Antigen Processing.



Adapted from - Cellular and Molecular Immunology **1** No.1; *Flutter,B. Gao,B.* **MHC Class I Antigen Presentation - Recently Trimmed and Well Presented**

Intuitively we would expect that the loading of peptides onto class I for antigen presentation must be a balance of two main factors. On the one hand, the peptides bound to class I should have a high enough binding affinity for the heavy chain alleles present, to avoid extensive peptide dissociation from class I at the cell surface. On the other hand, the class I molecules of the cell must be able to bind a large enough number of different peptide sequences, that the cell is able to present antigen from almost any cellular protein to passing T cells.

A correlation has been found between the length of time that class I molecules spend in the ER and the extent to which they are loaded with peptide implying that this balance must be carefully controlled (Lewis et al., 1996). This is probably one of a number of

functions carried out by the proteins in the PLC, which clearly has a role in the 'quality control' of class I exported from the ER. The fact that the majority of peptide free β_2m-heavy chain in the cell is found in the PLC, supports the theory that peptide binding almost certainly occurs in the PLC. In contrast, a much smaller proportion of peptide bound β_2m-HC is found in the PLC, suggesting that free β_2m-HC is recruited to the PLC and that peptide binding allows release from the PLC (Ortmann et al., 1994).

1.3.4.3 The role of TAP in the PLC

The PLC is centred on TAP, however TAP does not interact directly with heavy chain, but associates with it via tapasin. Although the presence of TAP in the PLC is topologically convenient (as it allows peptides to enter the ER in close proximity to heavy chain) it does not appear to be essential that TAP is present in the PLC for peptide loading. TAP mutant cell lines in which TAP does not associate with tapasin, still contain a PLC in which tapasin associates with heavy chain. When peptide is provided to this TAP-deficient PLC in permeabalised microsomes, class I molecules appear to become loaded with peptide and dissociate from tapasin in the normal way (Paulsson et al., 2001).

1.3.4.4 Tapasin

By immunoprecipitation of TAP1 it has been shown that class I heavy chain forms a complex with both TAP, and another molecule tapasin (TAP associated protein) (Androlewicz et al., 1994). The gene for tapasin maps to the class II region of the MHC close to the genes for TAP (Herberg et al., 1998), and the tapasin mutant cell line LCL721.220 (.220) shows significant defects in antigen presentation and the overall cell surface expression of class I is reduced (Greenwood et al., 1994). The mutation responsible for this defect is a simple base substitution 2 nucleotides from the 5` end of intron two of the gene coding for tapasin (Copeman et al., 1998). The GT nucleotide sequence, which is invariant in 5` splice sites, has thus been altered to GC, and as a result exon 3 splices directly to exon 1, excising exon 2 from the messenger RNA in the process. When wild type tapasin is reintroduced in to .220 cells the phenotype is rescued, cell surface expression of class I returns and the association between heavy chain and TAP is restored (Ortmann et al., 1997). It is apparent from this and other work that tapasin is required for interaction between the β_2m-HC heterodimer and TAP

(Sadasivan et al., 1996) (Ortmann et al., 1997) and this highlights the importance of tapasin in the formation of the PLC by acting as a bridge between TAP and class I.

Unlike in TAP negative cells, where the loss of cell surface expression arises from a lack of peptide translocation into the ER rather than from the absence of TAP in the PLC, it is essential that tapasin is both expressed and that it incorporates into the PLC to facilitate peptide loading. A mutant HLA-A*0201 class I heavy chain molecule known as T134K, in which threonine is replaced by lysine at position 134, is unable to interact with tapasin. As a result although T134K forms a heterodimer with β_2m in the normal way, it is not found in association with tapasin or TAP (Peace-Brewer et al., 1996). T134K traffics to the cell surface at an increased rate either in a peptide receptive state or with a low affinity peptide bound. Hence, it is unable to present endogenous viral epitopes to T cells (Lewis et al., 1996).

Since the discovery of tapasin and the demonstration of its importance in MHC class I antigen presentation, our understanding of its role has been complicated by the finding that cell surface levels of class I in human tapasin knockout cell lines are somewhat allele specific (Peh et al., 1998). Thus, whereas HLA-B*4402 is almost completely lost from the cell surface in .220 cells, HLA-B27 alleles seem to be normally expressed at the cell surface even in the absence of tapasin. This allelic difference has been pinpointed to residue 114 of the class I heavy chain (Park et al., 2003). Alleles with a histidine residue at position 114 express in the absence of tapasin, whereas alleles with a glutamic acid residue at position 114 are dependant on tapasin. Because of this, HLA-B27 and a number of other alleles, which still express at the cell surface in tapasin deficient cell lines, are often referred to as 'tapasin independent' alleles. However, it is interesting to note that even the HLA-B27 is found in the loading complex in normal tapasin proficient cells and hence, although it does not require tapasin association *per se* for cell surface expression, tapasin is still involved in its normal trafficking and may still play some role, for instance in the loading of certain peptide epitopes.

There are two main reasons postulated for the loss of surface class I in the absence of tapasin. One possibility is that class I cannot become properly loaded with peptide in the absence of tapasin and as a result does not traffic to the cell surface, but is degraded in the ER. This is supported by the finding that in .220 (tapasin deficient) cells, β_2m-HC heterodimers are retained for longer in the ER before trafficking or degradation (Tan et

al., 2002). Alternatively, it may be that class I heterodimers leave the ER as normal but that in the absence of tapasin, instead of loading with high affinity peptides, many of the class I molecules leave the ER loaded with low affinity peptides which dissociate easily either during trafficking or at the cell surface. When class I molecules become unloaded, heavy chain quickly dissociates from β_2m and the empty heavy chain molecules are internalised and marked for degradation: this would account for the low surface levels of class I. This latter situation appears to be the case in murine experiments where as expected, overall class I expression is low in tapasin knockout cells, but trafficking of K^b and D^b molecules measured by acquisition of endo H resistance is similar in spleen cells from tapasin$^{-/-}$ and tapasin$^{+/-}$ mice (Grandea, III et al., 2000;Garbi et al., 2000). It is likely that both mechanisms play a part in the low cell surface expression of class I in the tapasin mutant and the effect may vary from one class I allele to another.

An artificial soluble tapasin without transmembrane and cytoplasmic domains has also been used to rescue the deficiency of the .220 cell line. Although there is no association between TAP and class I in the rescued cells, cell surface levels of class I are restored (Lehner et al., 1998) supporting evidence from TAP mutant cells showing that TAP is not a necessary component of the PLC. However, Gao et al. have shown that although a similar, truncated form of tapasin restores normal class I surface expression on .220 cells, the peptides loaded on the surface of rescued cells are suboptimal. This indicates that the transmembrane and cytoplasmic fragments of tapasin involved in its association with TAP, are required for the full function of tapasin (Gao et al., 2004).

Interestingly a recent study comparing the peptide profile of HLA-B8 in tapasin proficient and tapasin deficient cell lines has found that contrary to expectation, the binding affinity of class I in the absence of tapasin is as high as or higher than in the presence of tapasin. The implication of this is that tapasin acts to facilitate the binding of a wider range of peptides, rather than acting to ensure the binding of only high affinity peptides. This gives rise to the view that rather than being a peptide editor for class I tapasin is a facilitator of peptide loading (Zarling et al., 2003).

1.3.4.5 ERp57

ERp57 is a member of the Protein Disulfide Isomerase (PDI) family with a MW of 57kDa, whose functions include disulfide bond isomerisation, reduction and oxidation (Gilbert, 1997;High et al., 2000;Ellgaard and Helenius, 2001). The catalytic activity of this family of proteins is dependent on the cysteine residues in 2 CXXC motifs (Hirano et al., 1995;Urade et al., 1997). ERp57 forms complexes with either calnexin or calreticulin, and together they ensure the correct folding and disulphide bond formation of monoglucosylated polypeptides in the ER (Oliver et al., 1999;Tan et al., 1997). It is not surprising then, that ERp57 is found with class I heavy chain in the early complex where calnexin is present, and also in the peptide-loading complex with TAP, class I, tapasin and calreticulin (Hughes and Cresswell, 1998;Lindquist et al., 1998;Morrice and Powis, 1998). There are two disulphide bonds within the folded heavy chain molecule one of which is found inside the peptide groove of class I. ERp57 is a likely candidate for the correct formation of these bonds, and there is now evidence that the presence of ERp57 within the complex may be crucial to correct peptide loading. It has been shown that ERp57 can specifically reduce partially folded peptide receptive MHC class I molecules while showing little or no activity for peptide loaded class I molecules (Antoniou et al., 2002). It is possible then that ERp57 may be important in the unfolding of MHC class I molecules, by reduction of heavy chain disulfide bonds when they remain in a peptide receptive state for a prolonged period, for example when peptide supply is poor. More interestingly, it was shown recently that ERp57 forms an intermediate in which tapasin is disulfide bonded to ERp57 (Dick et al., 2002). Mutation of cysteine residue 95 of tapasin to alanine, abolishes formation of this disulfide bond intermediate and also seems to prevent complete oxidation of class I. As a result the C95A tapasin mutant is only able partially to restore class I expression in .220 (tapasin deficient) cells compared with a full recovery when normal tapasin is transfected into these cells (Dick et al., 2002). The implication of this is that formation of this last disulfide bond of class I in the loading complex may require both ERp57 and tapasin, and that this step is somehow crucial in the quality control process of peptide loading onto class I. In 2006 work was published in which ERp57 knockout cells were studied, and this will be discussed further in chapters 3 and 7 (Garbi et al., 2006).

1.3.4.6 Calreticulin

The ER chaperone calreticulin has two well characterised basic functions. Firstly, it has a vital function in intracellular calcium homeostasis and as a result, deletion of calreticulin causes a lethal impairment of cardiac development during embryogenesis (Mesaeli et al., 1999). Secondly, it is an important ER chaperone in the folding of newly synthesised glycoproteins such as class I molecules. It has been established by immunoprecipitation that calreticulin is present in the PLC and that this interaction relies on the presence of TAP and tapasin (Sadasivan et al., 1996;Lewis et al., 1998;Hughes and Cresswell, 1998). However, the role of calreticulin in class I antigen processing is much less well characterised than that of tapasin or TAP.

The calreticulin deficient mouse fibroblast cell line K42 has allowed some assessment of the role of calreticulin in the PLC. Although loss of calreticulin does not appear to be as severe in antigen processing as a loss of tapasin, K42 cells show a 50-80% loss in cell surface expression of class I as compared with wild-type K41 cells. When either exogenous peptide or β_2m is added to K42 cells, surface expression is increased 4.5 fold compared to just 1.2 fold in K41 cells (Gao et al., 2002). This suggests that loss of class I surface expression in K42 cells is due to loss of unstable class I molecules at the surface, which can be rescued by high affinity peptides or by an excess of β_2m. Pulse chase analysis shows that in the absence of calreticulin, heavy chain traffics to the cell surface at an increased rate (Gao et al., 2002) rather like the mutant HLA-A*0201 allele T134K (Lewis et al., 1996). This increased rate of trafficking could result in poor peptide loading within the ER. There is further evidence that peptide loading within the ER is defective in K42 cells; conformation dependant antibodies show that a much greater proportion of class I molecules in the ER remains in the peptide receptive state in K42 than in K41 cells. Not only this, but impaired T cell recognition was observed for 3 out of 4 peptide epitopes presented by K42 cells. However, heavy chain is still found in association with TAP, tapasin and ERp57 in the loading complex of K42 cells, indicating that the PLC can still form in the absence of calreticulin (Gao et al., 2002). The exact role of calreticulin therefore remains unclear, but it may be that the presence of calreticulin in the loading complex stabilises interactions between the other molecules and consequently, that heavy chain is retained for longer allowing better optimisation of peptide binding. One other attractive possibility is that calreticulin chaperones peptides from TAP to the peptide groove of class I. Cross linking experiments have shown that calreticulin can bind to peptides delivered to the ER via

TAP (Spee and Neefjes, 1997). It is known that peptides are found bound to chaperones such as heat shock proteins in the cytosol, (Paz et al., 1999;Udono and Srivastava, 1993) and it is possible that calreticulin could fulfil a similar role within the ER.

1.3.5 Export of class I to the cell surface.

It has been convincingly shown that premature export of class I from the ER to the cell surface results in poor surface expression and reduced antigen presentation to cytotoxic T lymphocytes (Lewis et al., 1996;Gao et al., 2002). However, the mechanism for the export of class I from the ER is not entirely clear.

The Bulk Flow model of ER export postulates that ER resident proteins are maintained in the ER by means of retention and retrieval signals. Other proteins, which do not have such signal sequences, traffic from the ER by default to the Golgi (Wieland et al., 1987). Under this model, class I would be retained in the ER whilst it was in contact with the peptide-loading complex (PLC), because of the presence ER retention signals carried by chaperone molecules. Conversely, class I molecules that were not bound by chaperone molecules would exit the ER. Consequently the rate of export would depend on the rate of release of class I from the PLC. This would explain the poor loading of class I molecules that bind poorly to the PLC such as T134K mutants and in tapasin deficient cell lines.

However, several studies show that an increase in the rate of release of peptide loaded class I from the PLC, induced by provision of an excess of high affinity peptide, has no effect on ER to Golgi transport (Suh et al., 1996;Marguet et al., 1999;Spiliotis et al., 2000). Furthermore, it has also been shown that peptide loaded class I molecules accumulate at ER exit sites, and that they associate with a putative cargo molecule BAP31 (Spiliotis et al., 2000). These findings point towards a selective model of class I export involving recruitment of peptide loaded class I into COPII vesicles, which transport them out of the ER and to the Golgi network. In this situation the export is limited by the rate of incorporation of class I molecules into COPII vesicles and not on release of class I from the PLC. In this model it is still important that the PLC retain poorly loaded class I, since although the overall rate of export of loaded class I does not rise concomitantly with release of class I from the PLC, the ratio of poorly loaded to well loaded class I molecules will increase if the PLC does not retain class I molecules

until they are loaded with high affinity peptide. As a result the proportion of stable class I being released from the ER will fall, and surface expression of class I and antigen presentation will be reduced.

Paulsson et al. (Paulsson et al., 2002) propose a further level of export control where poorly loaded class I can be returned to the ER from the Golgi. In their work they show that tapasin is present in the Golgi body and that it is able to associate with both the β and γ chains of the coatamer COPI. The COPI coatamer mediates retrograde Golgi to ER transport (Harter and Reinhard, 2000) and could therefore provide a mechanism for class I molecules which have lost their peptide during ER to Golgi transport to be returned to the ER, where they can be reloaded with peptide. They argue that the build up of class I molecules in the ER in TAP mutant cells is not due to lack of export of class I from the ER to the Golgi, but results from the return of unloaded class I molecules from the Golgi to the ER. This hypothesis is supported by the observation that levels of class I in the Golgi body of TAP negative are similar to those in wild type cells (Baas et al., 1992).

1.4 Outstanding questions and approaches for studying MHC class I antigen processing

Much is now known about the mechanisms of peptide loading and MHC class I trafficking to the cell surface. However, there remain a number of interesting outstanding issues to address:

The association of calnexin with heavy chain does not seem persist after the binding β_2m in human cells (Sadasivan et al., 1996). However, there is no formal proof of the trigger for the release of calnexin. It is possible either that binding of β_2m to heavy chain destroys the interaction between heavy chain and calnexin, or that once heavy chain is bound to β_2m the interaction with calreticulin dominates and so calnexin is lost. At an early stage in the assembly of class I molecules, heavy chain associates with β_2m. Since the recruitment of β_2m to class I heavy chain occurs at the same time as association with calreticulin (Sadasivan et al., 1996), it is possible that calreticulin is required for the efficient formation of the β_2m-HC heterodimer. Alternatively, the pairing of β_2m and heavy chain may happen automatically or other molecular chaperones may facilitate the process.

Once formed, the class I β_2m-HC heterodimer traffics to the peptide-loading complex, where it picks up its peptide cargo. Although many of the components of the peptide-loading complex have now been characterised, the functions of chaperones such as calreticulin, ERp57 and tapasin in peptide loading remain unclear. Possible roles for these proteins include: the maintenance of the folded β_2m-HC class I heterodimer, disulfide bond isomerisation within the peptide groove of class I molecules, peptide editing, and controlling the release of loaded class I molecules from the peptide-loading complex. Finally, at the time this work began, little was known about the importance or function of ERp57 in MHC class I antigen presentation.

In chapter 3 attempts are made to suppress the expression of the chaperone ERp57 in a mouse cell line using RNA interference. The presence of ERp57 in the peptide-loading complex and its covalent interaction with tapasin imply that it may be important in class I antigen processing, but its function still remains unknown. Additionally it is known to be present in the 'early complex' and may play a role in the degradation of class I molecules that fail to load with peptide. By suppressing ERp57 it would be possible to assess its importance in class I peptide loading and its effect on the interactions between other proteins involved in class I assembly.

In chapters 4-6 MHC class I fusion constructs are used to investigate the roles of chaperone molecules in the folding of heavy chain, β_2m and peptide. Fusion proteins were made by joining together different components of the trimeric class I complex with flexible linker sequences as follows:

β_2m: (human) 'β_2m'.

PB: 'peptide - linker 1 - β_2m'.

BA: 'β_2m - linker 2 - HLA-A*0201'.

BB: 'β_2m - linker 2 - HLA-B*4402'.

PBA: 'peptide - linker 1 - β_2m - linker 2 - HLA-A*0201'.

Where 'linker 1' codes for GGGGGGSGGSGGSGG, 'linker 2' codes for GGGGSGGGGSGGGGS and 'peptide' codes for the HLA-A2 binding peptide GILGFVFTL derived from influenza virus matrix protein (Fig. 1.4). The BA construct was provided by Prof. W Bodmer (Oxford University, UK). Dr. Bin Gao created and expressed the fusion constructs PB, BA and PBA in HCT cells and created the BB construct from BA by replacing HLA-A2 with HLA-B*4402 (HLA-B*4402 was kindly provided by Dr. C Peh. Oxford University, UK).

These fusion proteins may be thought of as partially assembled class I molecules and as such can be used to investigate the folding of wild type class I molecules:

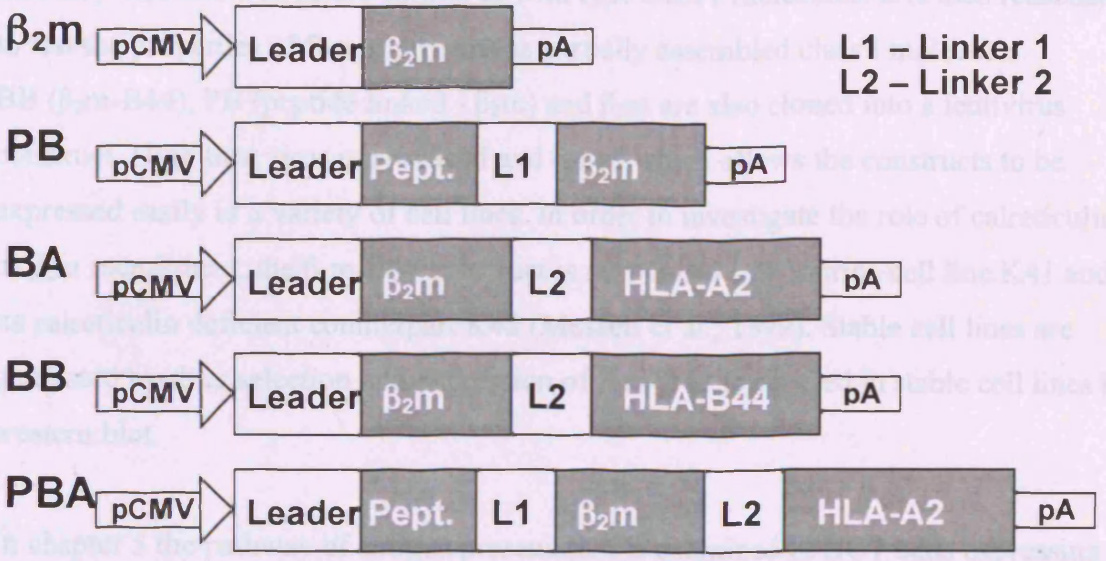
Fusion proteins in which β_2m is linked to heavy chain such as β_2m-A2 and β_2m-B44 will be used to investigate the interaction of human class I heavy with calnexin, and the function of chaperone molecules in the recruitment of β_2m. If the main function of calreticulin in class I antigen processing is the formation of the β_2m-HC heterodimer, then expression of β_2m-A2 or β_2m-B44 ought to be as strong in calreticulin deficient cells as it is in wild type cells. Conversely, the chaperone tapasin is thought to have a key role in peptide binding and so linkage of β_2m to heavy chain is unlikely to rescue the expression of class I in a tapasin deficient cell line such as .220.

The fusion protein PB (in which β_2m is linked to antigenic peptide) can be used similarly to investigate the process of peptide loading. Once PB is paired with HLA-A2 heavy chain, all 3 components of the fully folded class molecule have been brought together. If the binding of peptide to class I triggers release from the peptide-loading complex then the PB construct would not be expected to traffic the peptide-loading complex.

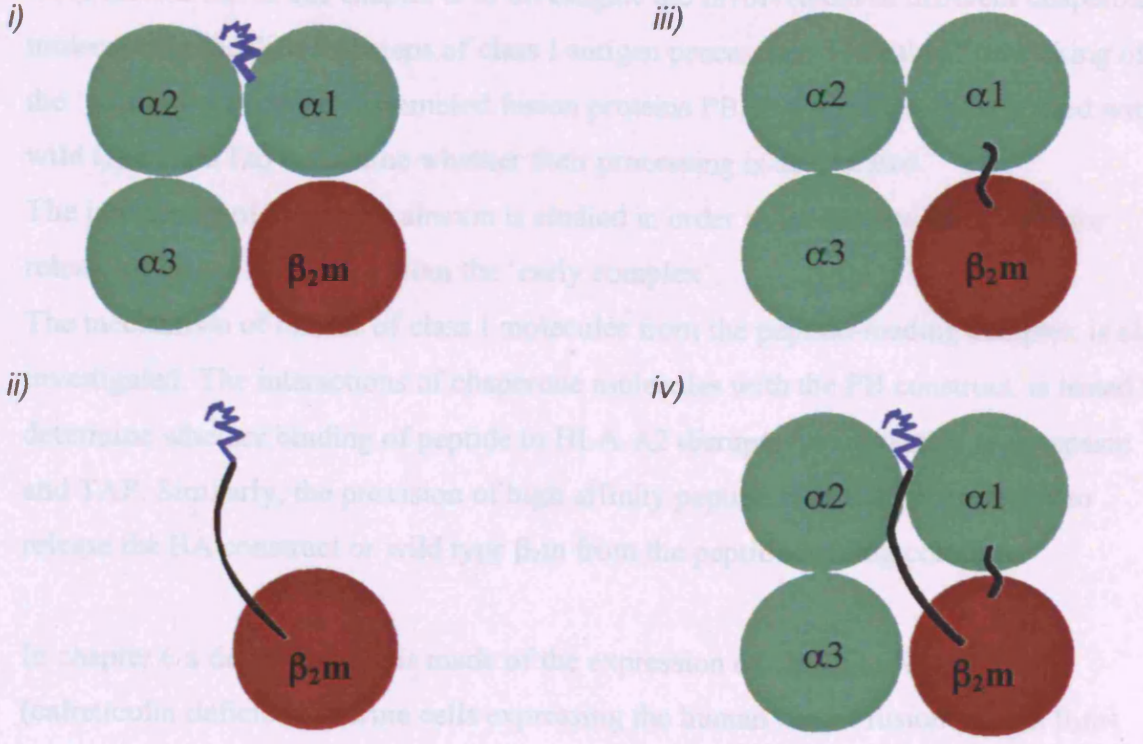
The fusion protein PBA takes this concept one stage further, since it already contains all three components of a fully assembled class I molecule. As a result it may have no requirement for chaperones.

Figure 1.4. Schematic representations of MHC class I fusion proteins.
a) Schematic representation of the genomic structure of class I fusion proteins. Leader – ER leader sequence, pCMV – promoter for transcription from cytomegalovirus, pA – polyadenylation signal. b) Schematic representation of the protein structure of class I fusion proteins. i) Native class I trimer, ii) peptide linked β_2m (PB), iii) β_2m-HC fusion proteins, iv) Single chain class I trimer PBA. β_2m – brown, heavy chain – green, antigenic peptide – blue, linker sequences – black.

a)



b)



The human colon carcinoma cell line HCT is β_2m deficient and as a result expresses no cell surface class I (Dexter et al., 1979). In chapter 4, β_2m and the class I fusion constructs PB, BA and PBA are expressed in HCT cells. The aim of this is to demonstrate that class I fusion constructs can rescue the phenotype of HCT cells in terms of: expression of cell surface class I and class I antigen presentation to T cells. If the fusion constructs can rescue class I expression in HCT cells then it can be assumed that they fold into a structure similar to wild type class I molecules. It is then reasonable to test the properties of fusion proteins as partially assembled class I molecules.
BB (β_2m-B44), PB (peptide linked - β_2m) and β_2m are also cloned into a lentivirus construct. High titre virus is produced and tested which allows the constructs to be expressed easily in a variety of cell lines. In order to investigate the role of calreticulin in β_2m recruitment, the β_2m-B44 construct is introduced into murine cell line K41 and its calreticulin deficient counterpart K42 (Mesaeli et al., 1999). Stable cell lines are generated by drug selection and expression of β_2m-B44 is verified in stable cell lines by western blot.

In chapter 5 the pathway of antigen presentation is examined in HCT cells expressing fusion constructs using immunoprecipitation and other techniques. The objective of the work carried out in this chapter is to investigate the involvement of different chaperone molecules in the different steps of class I antigen processing. The rate of trafficking of the 'partially assembled' assembled fusion proteins PB, BA and PBA is compared with wild type class I to determine whether their processing is accelerated.
The interaction of BA with calnexin is studied in order to investigate the trigger for release of class I molecules from the 'early complex'.
The mechanism of release of class I molecules from the peptide-loading complex is also investigated. The interactions of chaperone molecules with the PB construct, is tested to determine whether binding of peptide to HLA-A2 disrupts its interaction with tapasin and TAP. Similarly, the provision of high affinity peptide is tested for its ability to release the BA construct or wild type β_2m from the peptide-loading complex.

In chapter 6 a detailed study is made of the expression of class I in K41 and K42 (calreticulin deficient) murine cells expressing the human class I fusion protein β_2m-B44. β_2m-A2 is also expressed in these cells and compared to the expression of β_2m-B44. The aim of expressing these constructs is primarily to assess the importance of calreticulin in the recruitment of β_2m to class I heavy chain. But an assessment is also

made of the expression of these fusion proteins and of the compatibility of murine cells for the expression of human class I molecules.

2 General Methods

2.1 Cell Culture

2.1.1 Medium

Unless otherwise stated all cell culture growth medium was supplemented with Penicillin/Streptomycin 100Uml^{-1}/ 100μgml^{-1} (Life Technologies) and 10% Heat inactivated Foetal Calf Serum (FCS) (Insight Biotechnology, lot no. A01120-650).

2.1.2 Growth conditions

Unless otherwise stated all cells were grown at 37°C, 5%CO_2 in a humidified incubator (Jencons-PLS). Cells were cultured in sterile tissue culture flasks and plates were purchased from Corning.

2.1.3 Mycoplasma testing

All cell lines used were tested for Mycoplasma using either a fluorescence test and an agar culture test by Cancer Research UK, Clare Hall Laboratories (South Mimms, UK), or in house using a mycoplasma enzyme detection assay (MycoAlert). Cells were grown in the absence of antibiotics for 7 days before testing. Lines which were found to be positive for Mycoplasma were replaced by clean lines from Clare Hall laboratories or the ATCC where possible, or treated in house.

2.1.4 Mycoplasma treatment

Cell lines requiring mycoplasma treatment (HCT, K41 and K42) were cultured in the presence of 0.5μg/ml Mycoplasma Removal Agent (ICN) for 14 days or with BM-cyclin (Roche Diagnostics). Cells were then cultured for a further 28 days before retesting for mycoplasma.

Table 2.1. Cell lines

Cell Line	Description	Known class I related deficiency	Medium	Reference
HEK 293	Human Embryonic Kidney	None	D-MEM	(Graham et al., 1977)
HEK 293FT	Human Embryonic Kidney	None	D-MEM	Invitrogen – derivative of HEK 293
NIH 3T3	Mouse embryonic fibroblast	None	D-MEM	(Jainchill et al., 1969)
K41	Mouse fibroblast	None	RPMI 1640	(Mesaeli et al., 1999)
K42	Mouse fibroblast	Calreticulin deficient	RPMI 1640	(Mesaeli et al., 1999)
HCT	Human colon carcinoma	β_2m deficient	RPMI 1640	(Dexter et al., 1979)
NK92	Human Natural Killer-like cell line			(Gong et al., 1994)
CEM.NKR (NKR)	Human T cell	Calnexin deficient	RPMI 1640	(Howell et al., 1985)
K562	Human undifferentiated hematopoietic cell line	None	RPMI 1640	(Lozzio and Lozzio, 1975)
CTL_{FLU}	Human T cell clone.	None	T-media (see methods)	N/A. Obtained from Xiaoning Xu (Oxford, UK)
LCL 721.174 (.174)	Human B cell	TAP deficient and class II deficient	RPMI 1640	(Salter et al., 1985)
LCL 721.220 (.220)	Human B cell	Tapasin deficient	RPMI 1640	(Greenwood et al., 1994)
Daudi	Human B cell	β_2m deficient	RPMI 1640	(Klein et al., 1968)
BM9	Human B cell		RPMI 1640	European Collection of Cell Cultures.
TK143	Human osteosarcoma		RPMI 1640	(Rhim et al., 1975)

D-MEM (Dulbecco's Modified Eagles Medium) including Glutamax I, 4,500 mg/L D-glucose and 110mg/L sodium pyruvate (Gibco BRL) and RPMI 1640 including L-Glutamine (Gibco BRL) were supplemented with FCS and penicillin/streptomycin as indicated in Section 2.1.1.

2.1.5 Splitting adherent cell lines

Adherent cell lines were generally grown to around 90% confluence and then split by a factor of 2-10. In order to split cells, old medium was removed from the cells and the flask was gently washed twice with PBS (Gibco BRL). Trypsin/EDTA was added to cover the cells and incubated until the cells had lifted from the flask. An equal volume of medium containing FCS was added to quench the trypsin and the cells were centrifuged at 300g for 5 minutes, diluted and resuspended in an appropriate volume of fresh prewarmed medium and returned to culture flasks.

2.1.6 Splitting non-adherent cell lines

Non-adherent cell lines were split when necessary as determined by the colour of the medium and the density of cells. Typically cells were divided when they reached a concentration of around 10^6 cells/ml. 3-5 volumes of fresh medium was added to the medium containing cells. The diluted medium was then transferred to fresh culture flasks (Corning Life Sciences) of an appropriate size. Every 7-8 days or every third split, whichever was sooner; the cells were centrifuged at 300g and resuspended in fresh medium.

2.1.7 Counting viable cells

Cells were counted by adding a 10µl sample of cell suspension to 10µl of trypan blue 0.4% (Sigma). 10µl of this was then added to a counting chamber (Neubauer) and cells were counted under the microscope. The viable (unstained) cells in the 25 square field (0.1µl) were counted and the total multiplied by 20,000 to give the cell density in the original suspension in cells/ml. If counts were outside the range of 50-100 then the concentration of the cells suspension was adjusted and the count repeated to ensure accuracy.

2.1.8 Long term storage of cell lines

For long term storage, cell lines were preserved under cryogenic conditions in liquid nitrogen. Cells were counted, centrifuged and then resuspended at 10^7 cells/ml in FCS supplemented with 10%v/v Dimethyl Sulphoxide (Sigma). Cells were then divided into cryovials (Nunc) in 1ml aliquots and put into a freezing pot (Nunc) using isopropanol as

coolant. When placed in a -80°C freezer, the freezing vessel enables cells to cool at a rate of 1°C per minute. After 12-24 hours the cryovials were transferred to liquid nitrogen.

2.1.9 Thawing of frozen cells

Cryovials were removed from liquid nitrogen and warmed quickly in a 37°C water bath. Once thawed the cell suspension was added dropwise to 2ml of prewarmed 37°C FCS in a universal tube. The tube was topped up with medium and centrifuged at 300g for 6 minutes. Cells were resuspended in medium at 0.5 x 10^6 cells/ml and incubated overnight. The following day medium was replaced or cells split if necessary.

2.1.10 Cell Transfection

Cells were transfected using Lipofectamine 2000 (Life Technologies). Cells were seeded into 24-well plates 24 hours before transfection. Typically, on day of transfection, 1µl of lipofectamine was added to 50µl of Optimem 1 medium (Gibco-BRL) and incubated at room temperature for 5 minutes. 1µg of plasmid DNA or 2µl of T7 synthesised dsRNA (see section 3.2.3) was added to 50µl of Optimem 1. The Nucleic acid solution and the lipofectamine solution were mixed and incubated at room temperature for 20 minutes. Medium was removed from the cells, and cells were washed once in Dulbecco's PBS (Gibco BRL). Cells were then given 0.8ml of fresh Optimem 1 medium (with 10% FCS but without P/S). After incubation the lipofectamine nucleic acid mix (100µl) was added to the cells. Cells were incubated for 5 hours or overnight and the transfection medium was removed and replaced with normal growth medium.

2.2 *Fluorescence Activated Cell Sorting (FACS)*

2.2.1 FACS facility

FACS was carried out on a Becton Dickinson FACScan running CellQuest™ or Cell Quest Pro™ software. Cells were gated according to size as measured by forward scatter, and by light scattering properties as measured by side scatter. Typically 20,000 – 50,000 events were collected for each condition. FACS files were analysed either in Cell Quest™ or WinMDI.

2.2.2 Solutions

PBS: 1 PBS tablet (Oxoid) in 100ml ddH_2O.

FACS buffer: PBS, 1% FCS, 0.1% Sodium Azide (Sigma).

PERM buffer: FACS buffer, 0.1% saponin (Sigma)

PFA: 4g paraformaldehyde (Sigma) was dissolved (by heating in a fume hood to 65°C for 45 minutes) in 100ml PBS, allowed to cool and frozen in 2.5ml aliquots.

FIX buffer: FACS buffer, 1% formaldehyde (Sigma)

2.2.3 Preparation for Cell Staining

Cells were re-suspended to a concentration of 10^6 cells/ml in cold FACS buffer and 100µl aliquots were added to wells in a round bottomed 96-well plate (Merck). The plate was centrifuged at 300g for 5 minutes, the supernatant flicked off gently and the plate vortexed to loosen the cells.

2.2.4 Staining for cell surface markers

Cells were resuspended in 25-50µl of FACS buffer containing the appropriate antibodies at their optimal dilution (determined by antibody titration see table 2.1). Cells were incubated with antibody in the dark at 4°C for 30-60mins. Cells were then centrifuged at 300g for five minutes and the supernatant was discarded. The cells were then washed 2 times in FACS buffer by vortex, resuspension and centrifugation. Where necessary the cells were then resuspended in 25-50µl of FACS buffer containing

secondary antibody and incubated as above and then washed twice in FACS buffer. Cells were washed one final time in FACS buffer, resuspended in 100µl of FIX buffer and transferred to LP2 tubes (A1 Lab Supplies).

2.2.5 Staining for intracellular proteins

Prepared cells were resuspended in100µl of chilled PFA and incubated at room temperature for 10 minutes. Cells were washed twice in PBS and then once in PERM buffer. Antibody staining was carried out as in section 2.2.4 except antibodies were diluted in PERM buffer. After the final wash cells were resuspended in 100µl of FACS buffer and transferred to LP2 tubes.

Table 2.2. Antibodies for FACS

Antibody	Target	Conc., Dilution	Source; type	Conjugation	Reference / Obtained from.
W6.32	Human MHC class I with β_2m	5-10µg/ml (sup'n), 1/2	Mouse; mAb	None	(Parham et al., 1979)
BBM1	Human β_2m	5-10µg/ml (sup'n), 1/2	Mouse; mAb	None	(Brodsky et al., 1979)
DAKO p $\alpha h\beta_2m$	Human β_2m	EC staining 1/200. IC staining 1/1,000.	Rabbit; pAb	None	DAKO
BB7.2	HLA-A2	0.5mg/ml, 1/200	Mouse; mAb	None	(Parham and Brodsky, 1981)
ERp57	Mouse ERp57	Unknown, 1/100	Rabbit; pAb	None	Dr. B. Gao (UCL, UK)
α-mouse PE	Mouse IgG	Unknown, 1/200	Sheep; pAb	PE	Sigma
α-mouse FITC	Mouse IgG	1.1mg/ml, 1/400	Sheep; pAb	FITC	Sigma
α-rabbit PE	Rabbit IgG	200µg/ml, 1/50	Goat; pAb	PE	Caltag

N.B. Concentration of hybridoma supernatants (sup'n) was not tested but is normally in the range 5-10µg/ml.

2.3 SDS PAGE and Western Blotting

2x SDS loading buffer: (Tris-Cl pH 6.8, 4% w/v SDS (Sodium Dodecyl Sulphate), 0.2% w/v bromophenol blue (Sigma) and 20 % v/v glycerol)

5x SDS loading buffer – 10% SDS with other reagents as for 2xSDS loading buffer.

Lysis buffer: 1% Triton X-100 in PBS

1% digitonin lysis buffer: digitonin (Calbiochem) was dissolved in water at 5% w/v by boiling for fifteen minutes. Solution was stored at 4°C overnight and then filtered through 0.22μm PVDF filter (Millipore). Before use, solution was adjusted to 1% w/v in PBS and 10μl/ml of protease inhibitor cocktail (Sigma) was added.

Run buffer: 0.025M tris, 0.192M glycine, 0.1% SDS

Transfer buffer: 0.02M tris, 0.15M glycine, 20% methanol

PBST: PBS, 0.05% tween (Sigma)

Enhanced chemiluminescence (ECL) fluid (Pierce): 1ml super signal dura west luminal enhancer solution, 1ml stable peroxide buffer.

2.3.1 SDS Poly Acrylamide Gel Electophoresis (SDS-PAGE)

10^6 cells were centrifuged at 300g for 5 minutes, the supernatant discarded and the pellets were used immediately or frozen at -20°C for up to 2 weeks. Cells were resuspended in lysis buffer to a final concentration of 10^7 - 10^8 cells/ml and incubated on ice for 10 minutes. Cells were centrifuged at 13,000 rpm for 15 minutes at 4°C in a micro centrifuge (Eppendorf centrifuge 5415R) and the supernatant was added to an equal volume of 2x SDS loading buffer and heated to 94°C for 5 minutes. 10μl samples were added to the wells of 10-14% SDS protein separating gels made using Flowgen Protogel (Flowgen) and were run in an X-cell SureLock mini Cell System (Invitrogen) according to the manufacturer's instructions, for 1 hour at 200V or until samples had separated sufficiently. High Molecular Weight Rainbow Marker (Amersham) was run alongside the samples to allow analysis of the size of the detected protein.

2.3.2 Western Blotting

Following SDS PAGE the gel was blotted on to Hybond C Extra membrane (Amersham) using the X-cell SureLock mini Blot module (Invitrogen) at 20V for 2 hours. The membrane was carefully removed and blocked in 10 ml of PBS 5% Marvel (Premier International Foods) on a plate rocker (Stuart Gyro Rocker STR-9). The membrane was washed 3 times in PBS and transferred to 10 ml of PBST 5% Marvel containing the primary antibody. After 1 hour of rocking in primary antibody the membrane was washed 3 times in PBS for 1 minute and transferred to PBST 5% Marvel containing the secondary HRP conjugated antibody and rocked for 1 hour. The membrane was washed twice in PBST and twice in PBS. The membrane was dabbed dry with a paper towel and 2ml of enhanced chemiluminescence fluid was added evenly to the membrane. After 5 minutes the fluid was pipetted off and the membrane dabbed dry. The membrane was then wrapped in cling film and attached to one side of an exposure box with tape. Photographic film (Kodak Biomax) was exposed to the membrane for 10 seconds to half an hour. Film was developed by immersing in X-OMAT Developer for 30-60 seconds washing in water and fixing with X-OMAT fixing solution for 1 minute.

2.3.3 Immunoprecipitation

$1x10^7$ cells were counted and washed twice in chilled PBS (4°C). Cells were lysed by suspending in 1% digitonin lysis buffer and incubating on ice for 20 mins. Samples were centrifuged at 16,000g for 10 minutes and the supernatant was then added to the appropriate antibody. A small sample of lysate was kept for western blot. Samples were incubated for 1 hour with gentle mixing at 4°C after which, 40µl of protein G Agarose (Pierce) was added and the incubation was repeated. The formed protein G immunocomplexes were then washed 5 times in cold 0.1% digitonin. (Centrifugation was carried out at low speed 4,000g for 90 seconds to maintain the integrity of the protein G slurry.) Proteins were then eluted by adding 40µl of 5xSDS loading buffer, mixing thoroughly and heating to 94°C for 5 minutes. Samples were then centrifuged and used for SDS-PAGE and western blot immediately or stored for up to one week at -20°C.

Table 2.3. Antibodies for Western Blot and Immunoprecipitation

Antibody	Target	Source, conc. if known	Dilution for WB; volume for IP.	Species; Type	Conj.	Reference / Obtained from.
BBM.1	H β_2m	Hybridoma supernatant	1/2; N/A	M; mAb	None	(Brodsky et al., 1979)
BBM.1	H β_2m	Purified. 4mg/ml	N/A. IP 25µl	M; mAb	None	(Brodsky et al., 1979)
HC10	H class I heavy chain	Hybridoma supernatant	1/2; N/A	M; mAb	None	(Stam et al., 1986)
DAKO p αhβ_2m	H β_2m	Purified	1/1,000; N/A	R; pAb	None	DAKO
ERp57	Rat ERp57	Serum	1/2,000; N/A	R; pAb	None	Dr. B. Gao
CX	R calnexin	Serum	1/2,000; IP 40µl	R; pAb	None	Dr. B. Gao
CRT	R calreticulin	Serum	1/2,000; N/A	R; pAb	None	Dr. B. Gao
RB4	H TAP	Serum	N/A. IP 10µl	R; pAb	None	Dr. P. Wang
Tap A	H tapasin	Serum	N/A. IP 20µl	R; pAb	None	Dr. P. Wang
Rabbit Serum	None	Serum	N/A. IP 20µl	R; pAb	None	Dr. B. Gao
αm – HRP	M IgG	Serum	1/10,000; N/A	G; pAb	HRP	Sigma
RG96-HRP	R IgG	Purified	1/5,000; N/A	M; mAb	HRP	Sigma

H - Human, G - Goat, R - Rabbit, M - mouse, HRP - Horse Radish Peroxidase, mAb - monoclonal antibody, pAb – polyclonal antibody, IP – Immunoprecipitation, Conj. – conjugation. Where indicated antibodies were kindly provided by Dr. B. Gao (University College London, UK) or Dr. P. Wang (The Royal London School of Medicine and Dentistry, UK).

2.4 *Molecular cloning*

2.4.1 DNA extraction and purification

Plasmid DNA was extracted from *E. coli* strains DH5α and TOP10^{TM} (Invitrogen) using mini/maxiprep kits (Qiagen) according to the manufacturers instructions.

DNA fragments were purified from restriction endonucleases and other molecular biology reagents using a PCR purification kit (Qiagen).

DNA fragments were purified from agarose gels using Qiaquick Gel Purification kit (Qiagen)

DNA/RNA concentration and purity were assessed by measuring the light absorption at 260 and 280 nm using a spectrophotometer (Hitachi U1800). Concentration is calculated using the formulae:

$$\text{DNA concentration} = A_{260} \times 50\mu g/ml$$

$$\text{RNA concentration} = A_{260} \times 40\mu g/ml$$

Purity is assessed by calculating A_{260} : A_{280}. For DNA in low salt buffer pH 7.5 - 8 this ratio should be about 1.8. For RNA the ratio at neutral pH should be between 1.9 and 2.3.

2.4.2 DNA modifying enzymes

The following DNA modifying enzymes were used according to manufacturer's instructions.

Restriction endonucleases (Promega): Typically 1-2μg DNA was incubated for 1 hour with 1-2 units of enzyme, 5μl 10x reaction buffer, 0.5μl BSA and sterile water to a total of 50μl.

T4 DNA Ligase (Promega): Typically, plasmid and insert were mixed at a molar ratio of 1:3 and incubated overnight at 16°C with 2 units of enzyme and 1.2μl 10x reaction buffer in a total volume of 12μl.

Klenow DNA Polymerase large fragment (Promega). This enzyme was used to blunt unwanted overhanging single stranded DNA, which is a common product of restriction digests.

Calf Intestinal Alkaline Phosphatase (Promega). This enzyme was used to remove the activated phosphate groups at the ends of dsDNA fragments and is useful to avoid the self-ligation of blunt ended plasmids.

2.4.3 Agarose Gel separation of DNA/RNA

50x TAE: 2M Tris-Acetate, 0.5M EDTA pH 8.3 (Eppendorf)

Ethidium bromide stock solution: 1g EtBr in 100ml ddH_2O. (Stored in the dark at RT)

Working TAE buffer: 1/50 dilution of 50x TAE

1% agarose gel: 1g agarose (Sigma), 100ml TAE buffer. Heated by microwave and allowed to cool to 65°C before pouring.

2µl of 6x loading buffer (Promega) was added to 10µl of DNA/RNA sample. DNA/RNA samples were then run alongside a 1Kb Ladder (Promega) in TAE buffer through 1% agarose gels at 100V for 20-40mins. Gels were stained in 200ml TAE buffer with 10µl ethidium bromide stock solution and visualised using an AlphaImager system (Alpha Innotech).

2.4.4 Polymerase Chain Reaction (PCR)

Reagents for PCR were mixed in a total volume of 50µl as laid out in the table below. According to the desired product either PfU Ultra (Stratagene) or Taq (Invitrogen) DNA Polymerase was used. PCR was typically carried out for 30 cycles on a GeneAmp PCR System 9700 (PE Applied Biosystems):

1) Denaturing (initial) 94°C 5 mins.

2) Denaturing (cycle) 94°C 30 secs.

3) Annealing (cycle) 40-60°C (according to primers) 45-60 secs.

4) Polymerisation (cycle) 72°C 60-90 secs.

5) Polymerisation (final) 72°C 5 mins.

6) 4°C

Table 2.4. Reagents for standard PCR

Reagent	Stock Concentration	Volume (µl)	Final concentration
PCR buffer (Tris-Cl (pH 8.4), KCl)	10X (200mM, 500mM)	5	1x (20mM, 50mM)
$MgCl_2$	50mM	1.5	1.5mM
dNTP (Invitrogen)	Each at 5mM	2	0.2mM
Primer 1	12.5µM	1	0.25µM
Primer 2	12.5µM	1	0.25µM
DNA polymerase (Taq or PfU)	5U/µl	0.2 - 0.5	1-2.5 units
H_20		38 - 38.3	
Total		50	

2.4.5 Bacterial Growth

Sterilisation of broth and agar was carried out by autoclaving at 120°C for 90 mins.
Luria-Bertani (LB) broth – 16 LB capsules (Bio101) were added to 1 litre of ddH_2O and sterilised. (Final formulation – 10g tryptone, 5g yeast extract, 0.5g NaCl, 1 litre H_2O)
LB agar – 10g/litre of select agar (Gibco BRL) was added to LB broth before sterilisation. Agar was allowed to cool and was remelted by microwaving. It was then placed in a 45°C water bath to cool slowly before adding the appropriate antibiotic and pouring 15ml aliquots into 10cm agar plates (Sterlin).
SOB (Super Optimal Broth) - 20g tryptone, 5g yeast extract, 0.5g NaCl, 5g $MgSO_4.1H_2O$ (Bio101 formulation) in 1 litre distilled water.
SOC (Super Optimal Catabolite repression) - filter sterilised glucose was added to autoclaved SOB to a final concentration of 20mM
Stock ampicillin – 500µl aliquots of 50mg/ml ampicillin (Sigma) were kept at -20°C. Stock ampicillin was diluted 1/1000 into LB broth and LB agar as required.
Stock blasticidin - 500µl aliquots of 10mg/ml blasticidin (Invitrogen) were stored at -20°C.

2.4.6 Transformation of chemically competent E. coli

Competent cells were thawed slowly on ice (~30 mins) and gently resuspended. 100µl of cells was dispensed into a pre cooled eppendorf and ~10ng supercoiled plasmid DNA or 1-5µl of T4 ligation product was added. Cells were incubated with the DNA on ice for 30 mins before heat shocking for 30 secs at 42°C on a heat block (Grant QB2T). Cells were put back on ice and topped up with 0.5 ml of prewarmed SOC (37°C). Cells were shaken vigorously for 1 hour at 37°C to allow the bacteria to recover. 100 - 250µl of transformed bacteria was then spread on LB agar containing the appropriate antibiotic for selection. Agar plates were incubated overnight at 37°C.

2.5 T cell methods

2.5.1 T cell expansion

The CTL clone specific for influenza matrix protein derived peptide GILGFVFTL, was obtained from Xiaoning Xu (Institute of Molecular Medicine, Oxford). It was expanded *in vitro* in 2 week cycles as follows:

T-media: 50% Ex Vivo 15 media (Cambrex), 50% RPMI 1640 supplemented with 5% FCS, 5% Filter sterilised Human AB serum (Sigma) and 55μM 2-mercaptoethanol (Sigma).

Irradiated PBMCs: PBMCs were isolated from 3 donor Buffy Coat Residue samples (Colindale blood bank) by density centrifugation using Lymphoprep (Axis-Shield). Cells were counted and resuspended in 50ml of T-media at $4x10^6$ cells/ml and then irradiated with a dose of 25 Greys.

48 hours after thawing or 2 weeks after previous stimulation, T cells clones were counted and $1x10^6$ cells were suspended in 50ml T-media and mixed with 50ml irradiated PBMCs. T cells were stimulated with 40ng/ml monoclonal antibody OKT-3. 24 hours after stimulation media was supplemented with 200units/ml of IL-2. Subsequently, every 3 days half the media was removed and fresh T-media supplemented with 200units/ml IL-2 was added. Cultures were maintained at $1\text{-}2x10^6$ cells/ml throughout. For long term storage, cells were frozen at 12 days after stimulation.

2.5.2 Enzyme Linked Immuno-Spot assays (ELISpots)

ELISpot assays can be used to measure the number of cells in a population secreting specific factors such as IFNγ. IFNγ is a marker released by activated T cells, therefore an IFNγ ELISpot can be used to determine the number of T cells in a population that are activated to a given stimulus.

2.5.2.1 Plate preparation

96 well polyvinylidine difluoride (PVDF) (Millipore, Bedford, MA, USA) plates were coated for 2 hours at 37°C with 50μl per well of 15μg/ml anti-human IFNγ mouse IgG1 (clone D1K, Mabtech). This was diluted in PBS (w/o Mg and CaHCO3, GIBCO™,

USA) from the stock. Following incubation the wells were washed x8 in 200µl PBS 1% FCS in sterile conditions using a vacuum pump to aspirate (Vaccubrand, GmbH). 100µl complete medium (RPMI plus 10% v/v FCS) was then added to each well and incubated for 1 hour at 37°C to block.

2.5.2.2 Preparation of target cells or CTL stimulus.

When CTLs were to be stimulated by peptide pulsed target cells, the targets were washed and suspended in complete medium containing 10µM peptide at 1 x 10^6 cell/ml. Cells were mixed thoroughly with peptide and incubated at 37°C for 1 hour. After incubation cells were washed x8 in PBS 1% FCS to ensure no free peptide remained. Medium was aspirated out of the plate and 5 x 10^4 peptide pulsed target cells were added to each of the relevant wells in 100µl of RH10 (RPMI 1640 (Gibco BRL) with 10% Human AB serum (Sigma) and 100 units/µl IL-2). Stimuli other than target cells, such as peptide were added to wells at 2x final concentration in 100µl of RH10.

2.5.2.3 CTL stimulation

CTL were thawed into T-media (see section 2.5.1) + 100 units/ml IL-2 24 hours before the assay and incubated overnight at 37°C. On the day of the assay cells were re-suspended at 9 x 10^4, 3 x 10^4 or 10^4 cells/ml in RH10 + 10 units/ml IL-2. 100µl was transferred to each well. The plates were then incubated for 16 hours at 37°C. Following incubation the cells were discarded and wells were washed x8 in PBS as before.

2.5.2.4 Detection of activated T cells

After plate washing, 50µl of 1µg/ml biotinylated mouse IgG1 anti human IFNγ (clone 7-B6-1, Mabtech) was transferred to each well and the plate was then incubated for 2 hours at 37°C. The plate was then washed x8 with PBS and 50µl of 1µg/ml streptavidin alkaline phosphatase (Sigma) added to each well. The plate was incubated again for 1 hour at 37°C and was washed again x8 with PBS. For detection of cytokine production the wells were developed using 5-bromo-4-chloro-3-indolyphosphate and nitroblue tetrazolium (BCIP/NBT) substrate (BioRad). This was combined just prior to use as per the manufacturer's instructions and diluted in substrate buffer. 100µl of this was added per well and left at room temperature for 5 minutes or until dark blue spots appeared in

the wells. Running the plate under tap water for 3 minutes arrested the development of colour.

2.5.2.5 Phorbol 12-Myristate 13-actetate (PMA)

PMA (Sigma) is a Phorbol ester and a specific activator of protein kinase C. It is a good positive control for this assay as it stimulates release of IFN γ from T cells. Stocks of PMA were prepared in ethanol and stored at 1mg/ml at -20°C. PMA was used at a final concentration of 1μg/ml.

2.5.2.6 Assessment of developed plate.

After air-drying the developed plates over night at room temperature, the spots were counted using a video analyser (BioReader 3000, BioSys, GmbH). Pre-optimised reading methods, created by Dr. Katy Newton using the BioSys software were utilised. Triplicate or quadruplicate wells were read and the mean and standard deviation of the number of spots was determined for each condition.

2.5.3 T cell killing assays – (Chromium 51 release)

All work using the radioactive isotope Chromium 51 up to the T cell incubation was carried out in a designated room with appropriate lead shielding.

2.5.3.1 Target cell labelling

$5x10^5$ target cells, supplemented with 1μg/ml of peptide where necessary, were suspended in 50μl of normal growth medium in a 15ml falcon tube. 2MBq of Cr^{51} labelled sodium chromate (Amersham) was added and incubated in a water bath at 37°C for 1 hour with occasional gentle mixing.

Cells were washed 3 times in 10ml of normal growth medium and then resuspended in 10ml normal growth medium. (Final concentration of $5x10^5$ cells/ml).

2.5.3.2 T cell preparation

48 hours after thawing, or 10-14 days after stimulation, T cells were counted and resuspended in T-media at 3 different concentrations – typically $0.5x10^6$, $1.5x10^6$ and $5x10^6$ cells/ml. For each target cell to be tested T cells were then plated in a round well 96-well plate in triplicate. Triplicate wells were also set up for spontaneous release – 100µl T-media alone, and 100% release 100µl of T-media supplemented with 2% Triton X-100.

2.5.3.3 T cell – Target Cell incubation

100µl of the prepared target cells was then added to each well and mixed gently by pipetting. Cells were incubated together for 4 hours.

2.5.3.4 Measuring Chromium release

After incubation, plates were centrifuged at 1400rpm for 5 minutes. During centrifugation 175µl of Optiphase Supermix was added to each well of a scintillation plate (Perkin-Elmer). After centrifugation 25µl samples of supernatant were carefully removed and added to the scintillation plate. Plates were sealed and left for at least 15 minutes to allow mixing of the liquids. Plates were then gently vortexed to complete mixing and read on a beta scintillation counter (1450 Microbeta, Perkin-Elmer). Results were normalised for each target using the values for spontaneous release and 100% release.

3 Attempted suppression of ERp57 using RNA Interference

3.1 Introduction

3.1.1 ERp57 - An interesting target

In this chapter the technique of RNA interference is used to suppress the expression of Erp57 in order to study its functions in MHC class I antigen processing.
The ER resident protein disulfide isomerase ERp57 cooperates with the chaperones calnexin and calreticulin in the folding of polypeptides with monoglucosylated N-linked oligosaccharides (Oliver et al., 1999;Tan et al., 1997). Calnexin and calreticulin interact with class I at different stages of its development, but ERp57 is present in both the 'early complex' and in the peptide-loading complex (PLC) of MHC class I assembly (Hughes and Cresswell, 1998;Lindquist et al., 1998;Morrice and Powis, 1998). However, it is believed that Erp57 may have different functions at these 2 stages of development since the interactions it makes seem to be very different.

Human class I molecules in the 'early complex' can be immunoprecipitated with the monoclonal HC10 antibody and are not thought to be fully folded or associated with β_2m. These class I molecules have been shown to be disulfide bonded directly to ERp57 (Lindquist et al., 2001) and there is some evidence that its function in this early complex may be to fully reduce class I molecules that fail to fold, marking them out for retrotranslocation (Antoniou et al., 2002). In contrast, class I molecules immunoprecipitated with TAP or tapasin are not found to be disulfide bonded to ERp57. Instead Erp57 is now found disulfide bonded to tapasin and so its association with class I is probably indirect. Disruption of the interaction between tapasin and ERp57 by site directed mutagenesis of tapasin results in poor antigen processing for HLA-B44 and possibly other class I alleles (Dick et al., 2002). In 2002-3 when the work in this chapter was undertaken there was no published ERp57 gene knockout work. By using RNA interference it was hoped that the expression of ERp57 could be inhibited and more information about the exact functions of ERp57 could be obtained. If existing models were shown to be correct the suppression of ERp57 in a mouse β_2m deficient cell line would allow an analysis of its function in the early complex of class I maturation. Suppression would be expected to lead to inhibition of the retrotranslocation and degradation of free class I heavy chain, which usually occur in

these cells. If ERp57 cooperates with tapasin in peptide loading, then suppression of ERp57 in normal cells might lead to a phenotype similar to that in tapasin deficient cells. Surface expression of class I would be reduced and antigen presentation would be impaired especially for alleles such as HLA-B44, which is particularly reliant on tapasin (Peh et al., 1998;Dick et al., 2002).

In this work, ERp57 expression was targeted in mouse cell lines in the hope that a novel phenotype could be described, particularly in relation to MHC class I antigen processing. If successful this system would also allow ERp57 inhibition in the calreticulin deficient K42 cell line and by studying this "double knockout" it might be possible to assess the degree to which these molecules work cooperatively in MHC class I peptide loading.

3.1.2 Discovery of RNA interference

RNA interference (RNAi) is a sequence specific method of gene suppression, which acts at the level of translation by destroying messenger RNA (mRNA). In 1990, Jorgensen and colleagues tried to enhance the colour of petunias by introduction of additional pigment-producing genes into petunia (Napoli et al., 1990). To their surprise, instead of the expected deep purple colour, many of the flowers appeared patterned white or completely white. They named the observed phenomenon "cosuppression". At the same time, Alexander van der Krol et al. observed a similar phenotype when they introduced pigmentation genes into flowers; about a quarter of plants demonstrated reduced floral pigmentation, accompanied by a dramatic reduction in the expression of both the introduced gene and the homologous endogenous gene (van der Krol et al., 1990).

Guo and colleagues produced a similar loss-of-function or gene-knockout phenotype in *C. elegans* by direct injection of antisense siRNA (small interfering RNA) to the par-1 gene, and showed that a similar inhibitory effect on par-1 gene function could be elicited by the injection of sense siRNA. (Guo and Kemphues, 1995). In 1998, Fire and colleagues reported that double-strand RNA was more potent as a silencing trigger than either antisense or sense RNA alone (Fire et al., 1998). Following on from work showing that introduction of sense RNA could be as effective as antisense RNA in the suppression of gene expression; they used sense and antisense RNA both individually

and in synergy. They found that when sense and antisense RNA were used together the gene suppression was at least 100-fold higher than the individual use of either sense or antisense RNA. Not only this, but they also showed that only a few copies per cell of double stranded RNA were needed to suppress gene expression, which led them to suggest that the process was in some way catalytic.

Using plants cells where the analogous process of "Post Translational Gene Silencing" (PTGS) occurs, it was shown that mRNA could be degraded in the presence of short RNA molecules of 21-25 nucleotides (Hamilton and Baulcombe, 1999). In an *in vitro Drosophila* system it was then demonstrated that "21-22 nucleotide [dsRNA] fragments are the sequence-specific mediators of RNAi" (Elbashir et al., 2001a).

The natural use of RNA interference in higher organisms may be mainly as a defence against viruses rather than in cellular gene expression (Jensen et al., 1999) (Ratcliff et al., 1999). At some point within their life cycle, genetic elements such as transposons, and many viruses, such as those of the retrovirus family including HIV, produce dsRNA. Cells of higher organisms do not normally produce large pieces of dsRNA and hence have evolved to use of RNA interference as a viral defence mechanism, by destroying mRNA with sequence complementary to that of the double stranded RNA present (Jensen et al., 1999). Although naturally RNA interference may be primarily used as a viral defence mechanism, it can also be used effectively to suppress cellular gene expression making RNA interference an extremely useful tool in the study of gene function (Serwold et al., 2002).

3.1.3 Mechanism of RNA interference

In Drosophila large pieces of dsRNA are digested in an RNase III-like mechanism yielding 21-23 nucleotide dsRNA fragments with a 3' overhang of 2 nucleotides at each end (Elbashir et al., 2001a). The protein responsible for this cleavage is known as Dicer and contains both helicase and RNase activity (Bernstein et al., 2001). The 21-23bp dsRNA products are not randomly produced but rather, regular segments of dsRNA product are formed, which are able to guide cleavage of the corresponding mRNA at 21-23 nucleotide intervals.

The sequence specific degradation of mRNA relies on nuclease activity, this has been localised to the cytoplasm in a complex known as RISC (RNA induced silencing complex). RISC consists of both protein and a short piece of RNA (Bernstein et al., 2001) derived from the dsRNA. RISC somehow picks up the short fragments of dsRNA

produced by Dicer and uses them to capture complementary mRNA and then degrade it. It is known that Dicer digests long pieces of dsRNA into regular fragments of around 21-23bp and it has also been noticed that the digestion pattern of mRNA occurs at intervals of 21-23 nucleotides strengthening the link made between the two processes. RISC cuts the mRNA, 11 or 12 nucleotides along the complementary RNA that it holds in the complex. It is not currently clear whether the RNA guiding cleavage is still double stranded at this point or whether the complex simply retains one strand of the dsRNA.

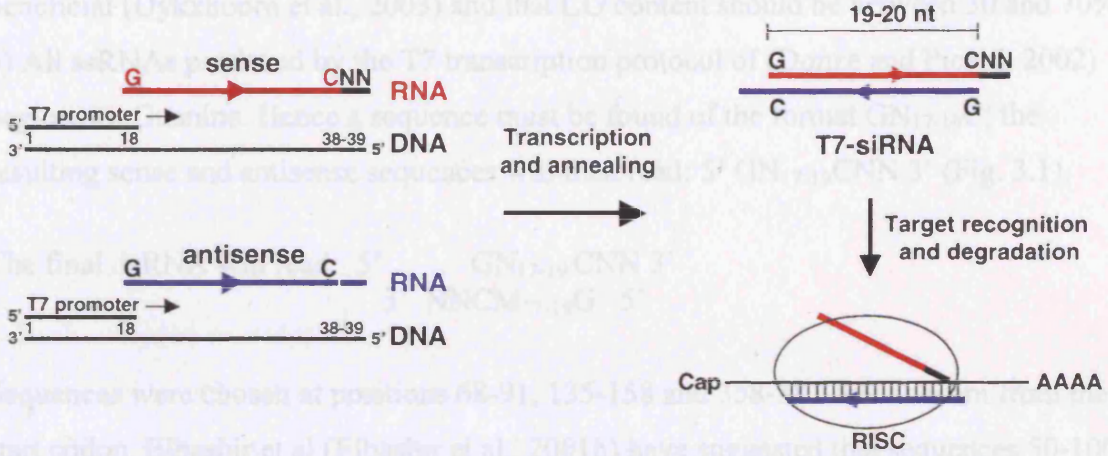
3.1.4 *In vitro* synthesis of dsRNA for RNA interference

To avoid the problems of activation of the interferon system by long pieces of dsRNA in mammalian systems it is better to use small pieces of dsRNA or small interfering RNAs (siRNAs) which can be used directly by RISC. This also has the advantage that it does not rely on the Dicer activity of the cell, which has been shown to be markedly lower in mammalian cells than in Drosophila. However, the difficulty with this is that it provides the RISC with only one species of dsRNA to act as a template and hence there is no certainty that the sequence of RNA chosen will suppress the gene effectively. In order to increase the chances of a successful gene knockdown, in this work three sequences were chosen against ERp57, and one published sequence which suppresses eGFP (Caplen et al., 2001) was chosen in order to demonstrate the viability of the technique.

A system of generating siRNAs has been demonstrated using T7 RNA polymerase to synthesise the two strands of RNA individually from a DNA template before annealing them to make the complete siRNA (Donze and Picard, 2002). The advantage of using T7 RNA polymerase is that the T7 promoter is very short and requires no downstream elements. As a result the DNA required consists simply of a short dsDNA sequence comprising the 18 nucleotide T7 promoter followed by the 21-23 nucleotide reading strand for RNA synthesis. DNA oligonucleotides were obtained from commercial sources, (Sigma-Genosys) and annealed to make such templates, and by designing the template sequences correctly 21-23 nucleotide RNA molecules were produced with complementary sequences containing 2 base 5' overhangs necessary for efficient RNAi (Fig. 3.1). In this way large quantities of several dsRNA were synthesised to allow screening of different sequences within the target mRNA. The siRNA against GFP of

the same sequence used is now also available commercially (Dharmacon, USA), allowing comparison with the sample prepared in our laboratory.

Figure 3.1. T7 Transcription of dsRNA. Schematic representation of *in vitro* synthesis of dsRNA.



Adapted from - Nucleic Acids Research **30** No. 10; *Donzé,O. Picard,D.* **RNA interference in mammalian cells using siRNAs synthesized with T7 polymerase**

3.2 Materials and Methods

3.2.1 Handling of RNA

When using RNA, commercially produced RNase free plastic (Fisher) was used wherever possible and all solutions and reagents used in the preparation of RNA were RNase free. A dedicated set of Gilson pipettes was used for RNA work. Two pairs of latex gloves were worn during work involving RNA.

3.2.2 Design of dsRNA sequences

Little is known about the best target sequences to use for RNA interference in mammalian cells. For this reason three different sequences were designed in the hope that at least one sequence would effectively inhibit ERp57 gene expression. The following criteria were taken into account in dsRNA sequence design:

1) The sequence should be homologous with part of the open reading frame of the gene of interest (i.e. ERp57).

2) The sequence should not be homologous to any other known mRNA sequence in the mouse genome. (A BLAST search was performed to check putative target sequences.)
3) DsRNAs should be 19-22 nucleotides long and have a 3' overhang of 2 nucleotides at each end, as this corresponds to the species created by Dicer protein (Elbashir et al., 2001b).
4) It has been suggested that an even distribution of the different nucleotides may be beneficial (Dykxhoorn et al., 2003) and that CG content should be between 30 and 70%.
5) All ssRNAs produced by the T7 transcription protocol of (Donze and Picard, 2002) begin with Guanine. Hence a sequence must be found of the format $GN_{17-19}C$; the resulting sense and antisense sequences will then read: 5' $GN_{17-19}CNN$ 3' (Fig. 3.1).

The final dsRNA will read: 5' GN_{17-19} CNN 3'
3' NNCM_{17-19}G 5'

Sequences were chosen at positions 68-91, 135-158 and 358-381 downstream from the start codon. Elbashir et al (Elbashir et al., 2001b) have suggested that sequences 50-100 bases downstream of the start codon are good targets; therefore one sequence has been chosen in this region. Other groups have not been able to confirm such an association and so the other sequences were chosen from other positions in the coding region of the mRNA. (Dykxhoorn et al., 2003)

3.2.3 Synthesis of double stranded RNA (dsRNA) with T7 RNA polymerase

Sense and antisense RNA molecules were synthesised individually and then annealed to produce dsRNA molecules as described (Donze and Picard, 2002).

3.2.3.1 T7 RNA polymerase template preparation

Desalted DNA oligonuceotides for the T7 recognition sequence (5'-TAATACGACTCACTATAG-3') and for its complementary sequence, followed by template for RNA synthesis (e.g. GFP sense 5' as in Caplen et al. (Caplen et al., 2001). 5'-ATGAACTTCAGGGTCAGCTTGCTATAGTGAGTCGTATTA-3'), were synthesised commercially (Sigma-genosys, St. Louis, Missouri, USA). 1nmol of the T7 recognition sequence was mixed with 1nmol of template DNA in 50µl of RNase free

water. The DNA was then annealed by heating the solution to 95°C on a heat block. After 2 minutes the block was switched off and the reaction was allowed to cool slowly.

3.2.3.2 Transcription

A 50µl reaction mix was prepared containing 1x T7 Transcription buffer, 1mM rNTPs, 100U of T7 RNA polymerase (Promega) and 200pmol of dsDNA (10µl of sample prepared above). The samples were incubated at 37°C for 2 hours. DNA was then removed by adding 1U of RNase free DNase (Promega) and incubating at 37°C for 15 minutes.

3.2.3.3 RNA annealment

Individually synthesised crude preparations of sense and antisense RNA were mixed and annealed by heating to 95°C for 5 minutes followed by incubation at 37°C for 1 hour. The mixture was adjusted to 0.2 M sodium acetate pH 5.2 and precipitated with 2.5 volumes of ethanol. The sample was then centrifuged, washed with 70% ethanol, dried and resuspended in 50µl of nuclease free water. RNA concentration and purity were quantified by spectrophotometry.

3.2.3.4 RNA interference of GFP (used as control)

Transfection of human embryonic kidney (HEK) 293 cells and mouse fibroblast NIH3T3 cells was optimised using a GFP expressing plasmid. To assess the effectiveness of the synthesised dsRNA, cells were simultaneously transfected with a plasmid expressing GFP and with either dsRNA against GFP or irrelevant dsRNA, using lipofectamine 2000 (see section 2.1.10). Fluorescence was observed using an Olympus 1X70 microscope and images were taken with an Olympus Vamedia C-3040 digital camera attached via C-3040-ADU and U-CMT adaptor units. Fluorescence was also detected by flow cytometry (see section 2.2).

3.2.3.5 RNA interference of ERp57

NIH3T3 Cells transfected with the 3 dsRNA sequences against ERp57 or against the irrelevant GFP were assessed for expression of ERp57 by Western Blot and by intracellular FACS.

3.3 Results

3.3.1 Good quality dsRNA is synthesised by T7 RNA Polymerase

Sense and antisense RNA sequences were synthesised by T7 polymerase and annealed to produce dsRNA and assessed by spectrophotometry. The four sequences of dsRNA produced were shown to be of good yield – between 9 and 25 mg/ml (Table 3.1). The high ratios of 2.3 between the absorbance at 260nm and 280nm suggest good purity, with little or no contamination with DNA. Sequences I, II and IV target sequences in ERp57, whereas sequence III is the sequence directed against GFP and used as a control.

Table 3.1. Concentration of synthesised T7 dsRNA

RNA sequence	Dilution; A_{260}	RNA concentration	A_{280}	A_{260}:A_{280}
I	1/900 ; 0.677	25 mg/ml	0.293	2.3
II	1/400 ; 0.570	9 mg/ml	0.245	2.3
III	1/400 ; 0.683	11 mg/ml	0.299	2.3
IV	1/400 ; 0.807	13 mg/ml	0.348	2.3

3.3.2 RNA interference suppresses GFP expression in HEK293 cells.

HEK293 cells were used to show the effectiveness of the RNA produced. These cells transfect easily, with at least 90% of cells expressing GFP after transfection of GFP expressing plasmid (Fig. 3.2). HEK293 cells were simultaneously transfected with GFP expressing plasmid, and either T7 dsRNA III or a commercially available dsRNA sequence against GFP (Dharmacon, USA). Both of these dsRNAs contain the same sequence complementary to the mRNA of GFP and would be expected to suppress the expression of GFP by destroying the mRNA. Effective suppression of GFP was seen with both these dsRNA sequences (Fig. 3.2). Suppression with Dharmacon dsRNA (commercial) was less marked than with T7 dsRNA, but it should be noted that the RNA concentration used was over 10-fold lower. Dharmacon dsRNA was used at this concentration according to the manufacturer's instructions; T7 dsRNA was used according to the methods outlined by Donze and Picard (Donze and Picard, 2002).

An incident laser at 488nm causes GFP to emit green light with a peak at 508nm. This can be detected on the FACS machine through the FL-1 channel (530/30 nm) or less strongly in the FL-2 channel (585/42 nm). FACS analysis of HEK293 cells (Fig. 3.3) shows that over 99% of cells transfected with GFP plasmid are emitting light in the FL-1 channel (b) compared to less than 1% of cells treated with lipofectamine alone (a). GFP is therefore expressed in at least 98% of GFP plasmid transfected cells. In contrast when cells are simultaneously transfected with GFP expressing plasmid and T7 dsRNA III less than 5% of cells express GFP (c). Treatment with irrelevant T7 dsRNA II caused a slight reduction in GFP expression with 97% of cells now showing expression of GFP (d).

The suppression of GFP in this experiment shown by the results in Figures 3.2 and 3.3 validate the technique. However, it would have been preferable to suppress an endogenously expressed protein. A cell line stably expressing GFP could have been used for this purpose, however, such a line was not available in the laboratory and since the final aim was to suppress ERp57 it did not seem worth investing the time and resources into the development of a stable line expressing GFP.

Figure 3.2. GFP detected by microscopy in transfected 293 cells can be silenced by both commercial and T7 preparation of dsRNA.
Cells were grown to 90 % confluence and then transfected with a) 1μg GFP plasmid, b) 1μg GFP plasmid and 2μg commercial dsRNA against GFP or c) 1μg GFP plasmid and 2μl (22μg) T7 dsRNA sequence III against GFP using lipofectamine 2000. 24 hours later GFP expression was detected by light microscopy using UV light excitation. Phase contrast pictures and UV light images are shown for purposes of comparison; the microscope magnification of images is x100.

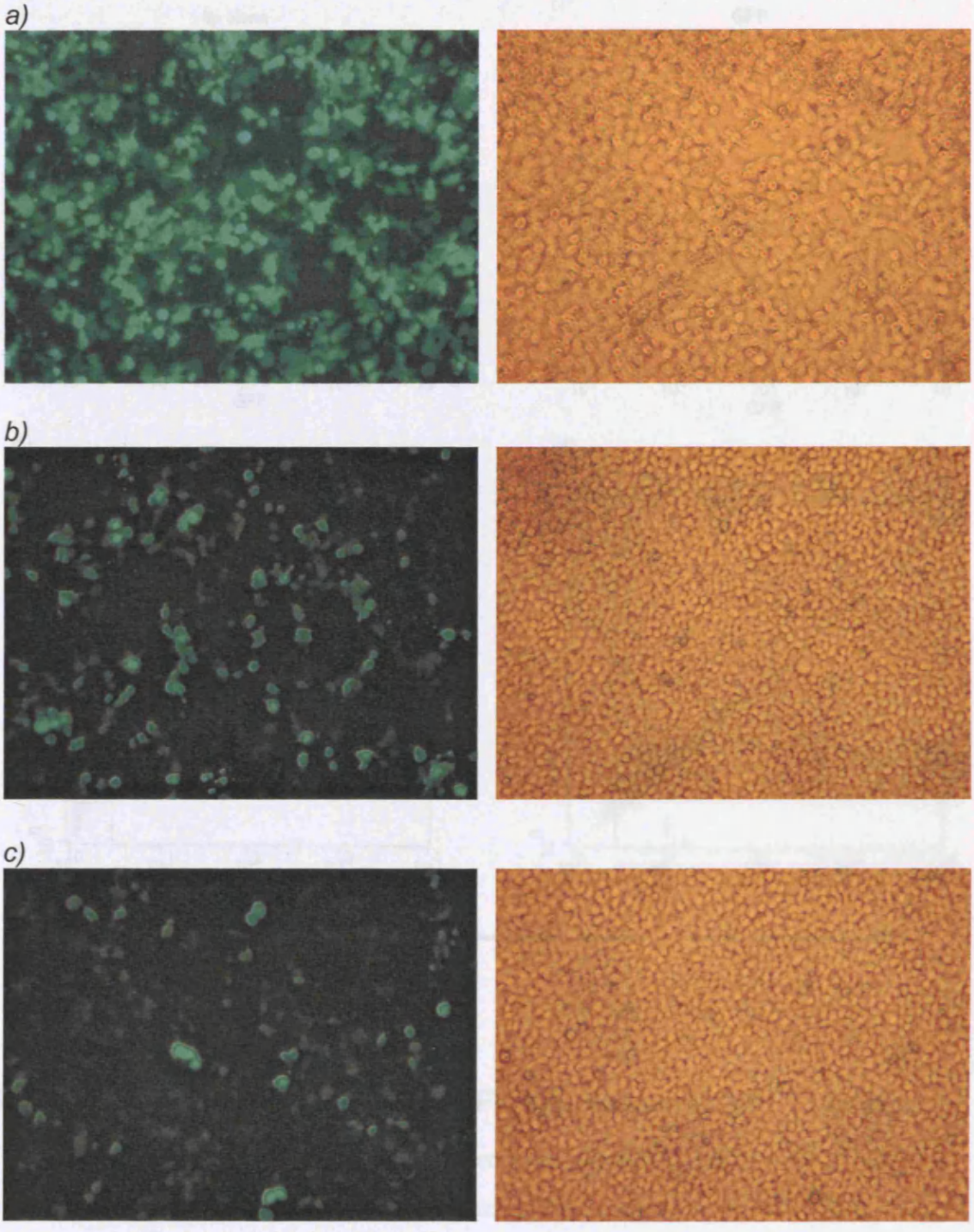
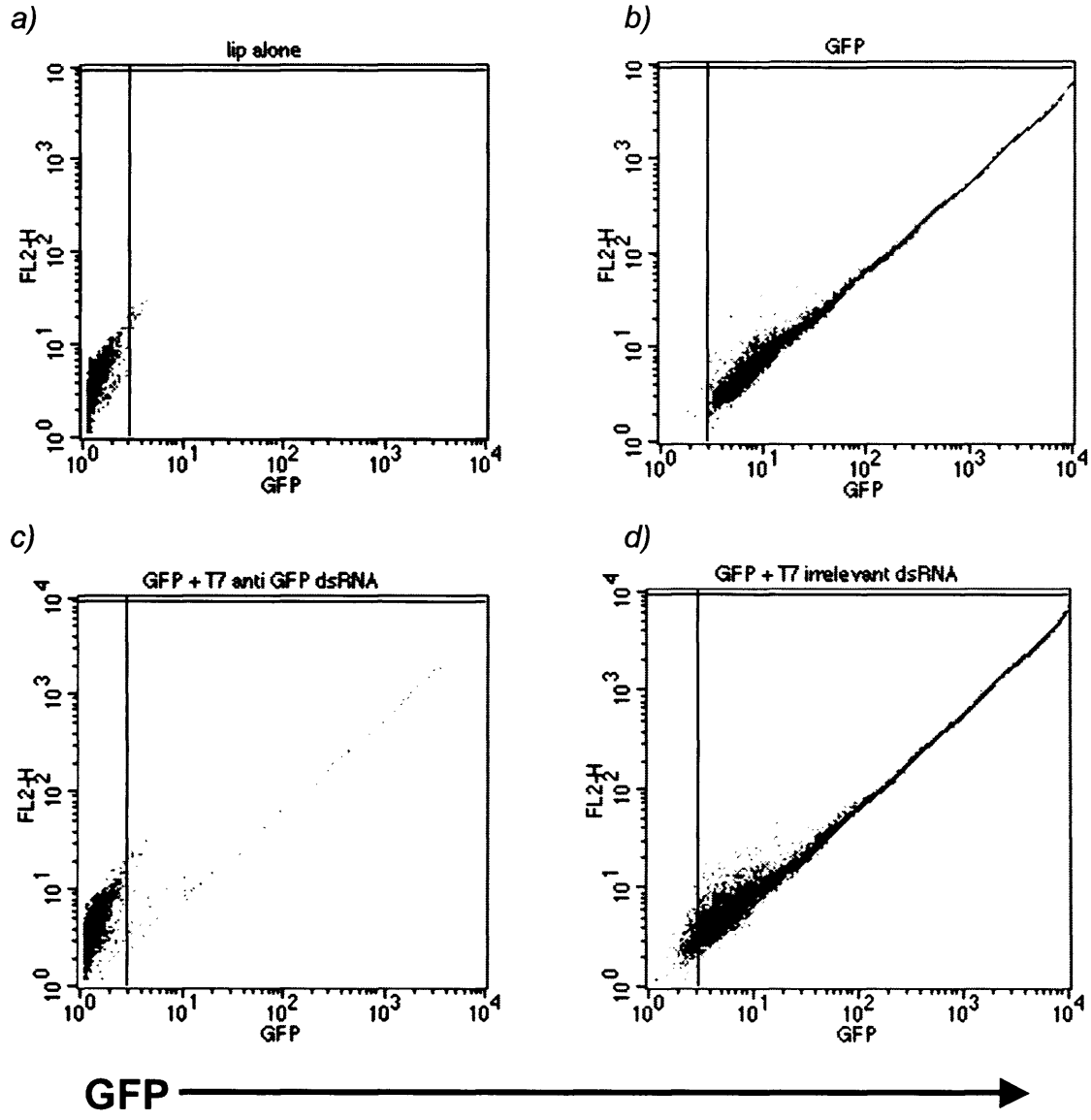


Figure 3.3. GFP expression detected by FACS is silenced by specific dsRNA.

HEK293 cells at 90% confluence were transfected using lipofectamine 2000 and assessed for expression of GFP by FACS using the FL-1 channel. GFP can also be detected in the FL-2 channel as can be seen from the data. a) Lipofectamine alone. b) 1µg GFP plasmid. c) 1µg GFP plasmid and 2µl of anti GFP T7 dsRNA (III). d) 1µg GFP plasmid and 2µl of irrelevant T7 dsRNA (II).



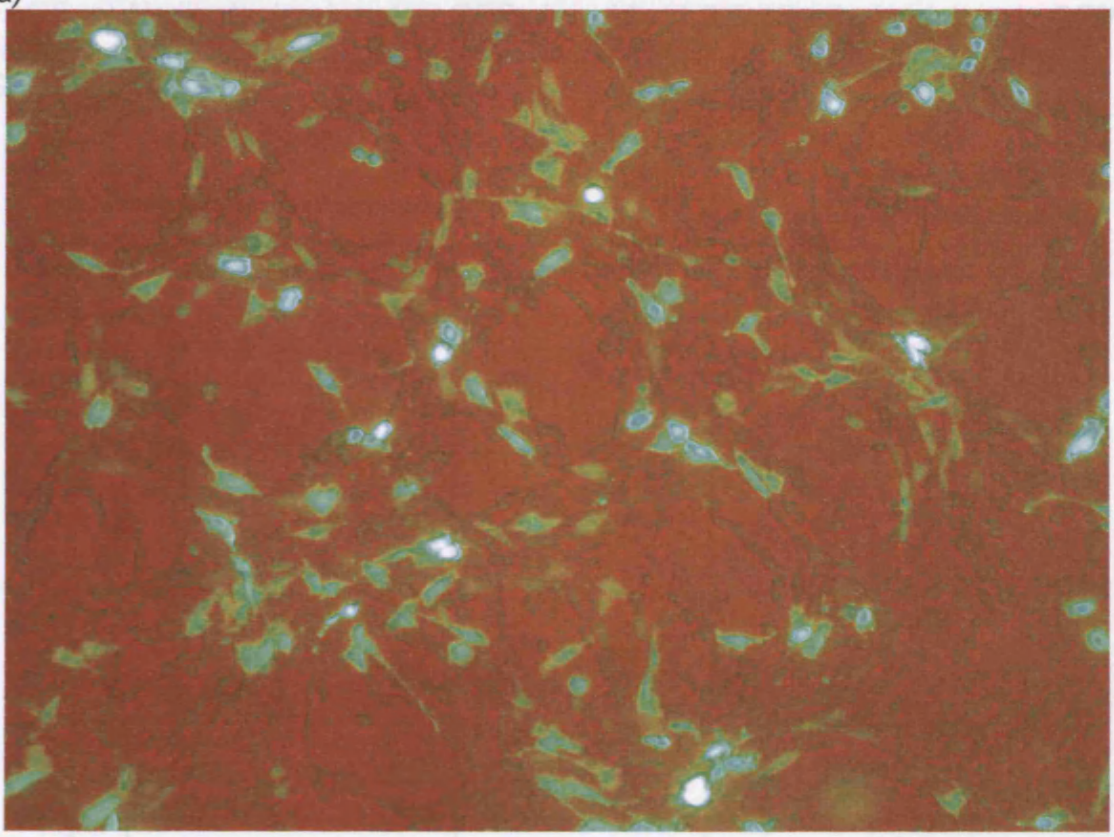
3.3.3 RNA interference silences GFP expression in NIH 3T3 cells.

To test the effectiveness of the T7 dsRNA produced against mouse ERp57 it was necessary to move into a mouse cell line. The mouse fibroblast cell line NIH 3T3 was chosen as a suitable line. When transfection of GFP plasmid and dsRNA against GFP were assessed in this cell line, cells transfected with GFP plasmid were found to express

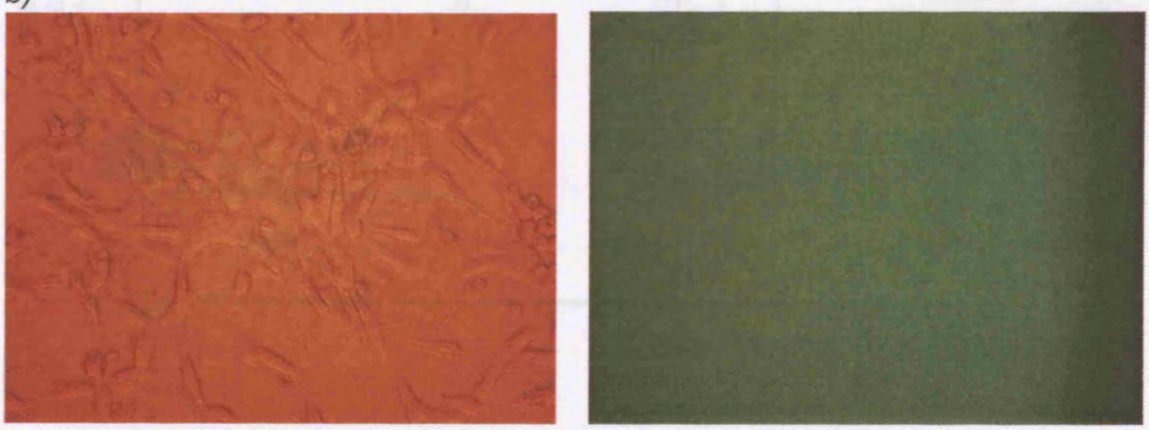
GFP. However there was no detectable fluorescence in cells transfected simultaneously with GFP plasmid and T7 dsRNA (Fig. 3.4), showing good silencing by dsRNA.

Figure 3.4. Transfection efficiency is low but T7 dsRNA silences GFP expression in NIH 3T3 cells.
a) NIH 3T3 cells were transfected with GFP alone and assessed by microscopy. Image shows both phase contrast and UV light excitation of GFP. b) NIH 3T3 cells were transfected with GFP and T7 dsRNA against GFP. 24 hours later cells were observed by microscopy. Left – Phase contrast image. Right – UV light excitation of GFP (none detected).

a)



b)



Although T7 dsRNA still effectively silenced GFP expression, the transfection efficiency of NIH 3T3 cells was considerably poorer than in 293 cells. In order to assess the effectiveness of dsRNA against T7 RNA it was felt that higher transfection efficiency was needed. Optimisation of conditions was performed by varying the lipofectamine concentration (Fig. 3.5) and the confluence of cells (Table 3.2).

Figure 3.5. NIH3T3 cells were transfected most effectively with 5µl lipofectamine/ '6 well'.
Transfection efficiency of GFP plasmid, in 3T3 cells grown to 20% confluence in a 6-well plate, was optimised for lipofectamine concentration and analysed by FACS. a) No lipofectamine, 1µg GFP plasmid. b) 2.5µl lipofectamine, 1µg GFP plasmid. c) 5µl lipofectamine, 1µg GFP plasmid. d) 10µl lipofectamine, 1µg GFP plasmid.

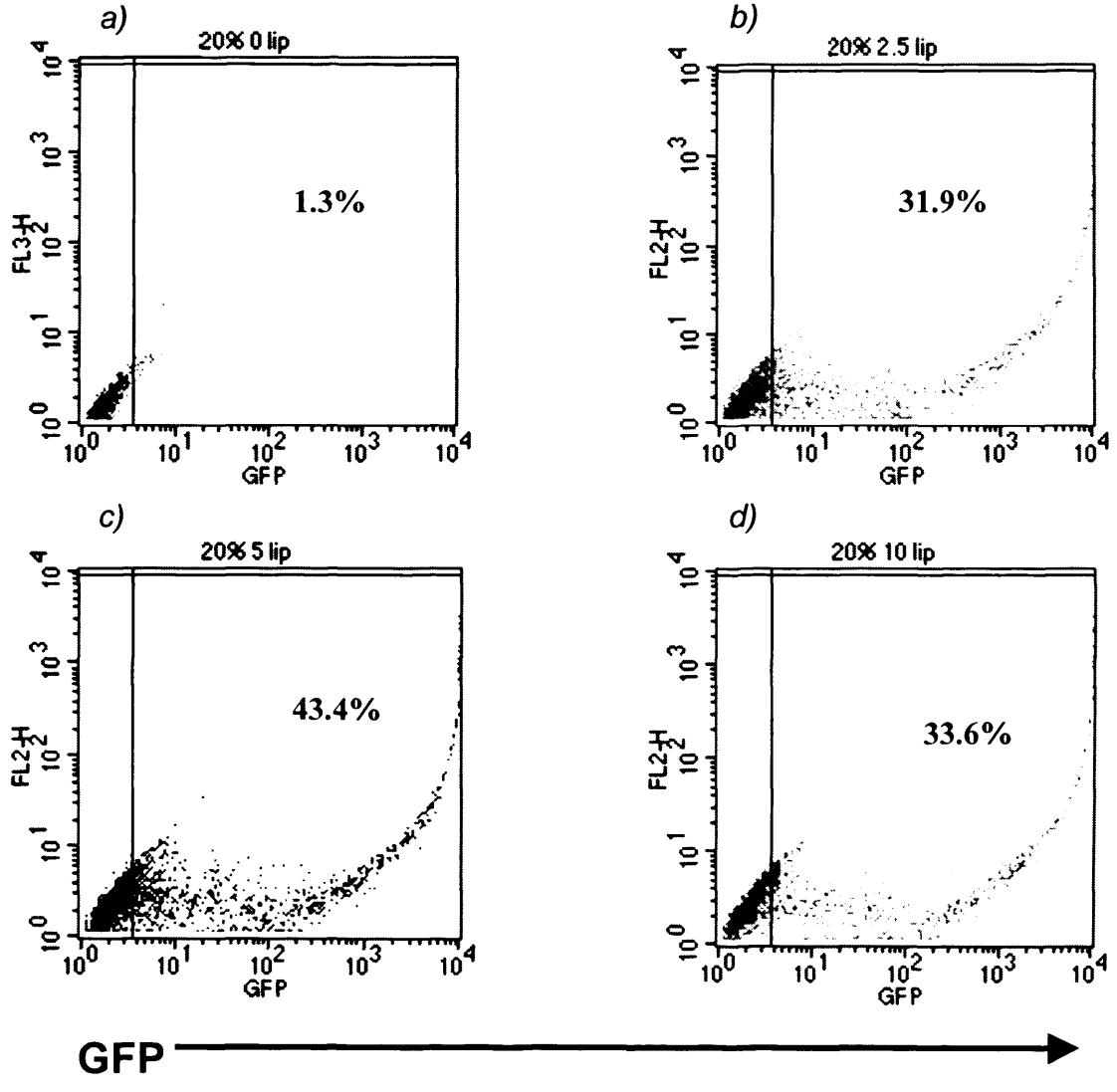


Table 3.2. NIH 3T3 cells were transfected more effectively at low confluence.

Cell confluence on day of transfection	Amount of GFP plasmid used	Percentage GFP positive cells by flow cytometry
100%	None	3.19
100%	1μg	19.62
90%	1μg	26.00
60%	1μg	31.55
30%	1μg	43.53
20%	1μg	43.69

Based on these results the optimised conditions for transfection of NIH3T3 cells were: cells at 20-30% confluence on day of transfection and 5μl of lipofectamine per well in a six well plate. Transfection efficiency under these conditions was typically between 38 and 45%.

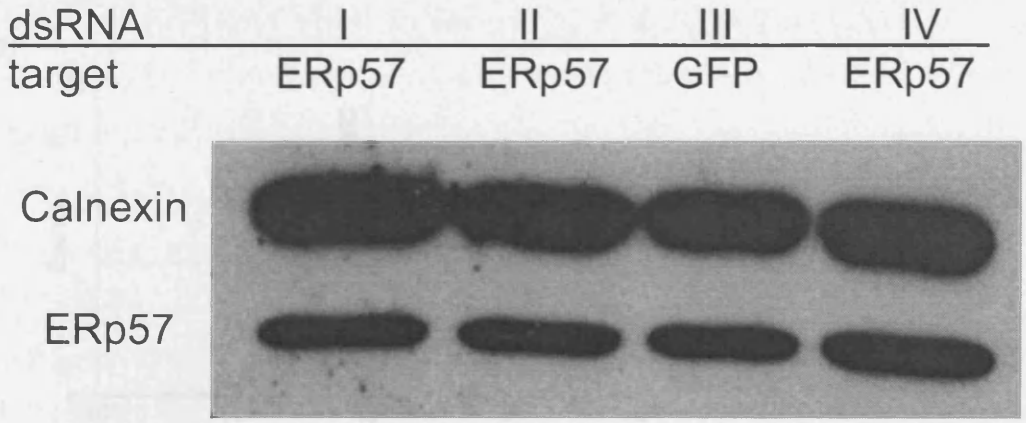
Although efficiency of plasmid DNA transfection does not necessarily correlate exactly with the efficiency of dsRNA transfection, in these experiments RNA interference with T7 RNA III invariably suppressed GFP expression to levels undetectable by blue light microscopy. It is reasonable to assume then that the transfection efficiency of the dsRNA must be at least as good as that for plasmid DNA since dsRNA is suppressing expression of GFP in every cell which has received plasmid.

3.3.4 The sequences chosen for RNA interference do not silence expression of ERp57 effectively.

In order to assess the suppression of ERp57 by RNA interference, the ERp57 protein level was measured by western blot against the housekeeping gene calnexin (Fig. 3.6). By western blot it was not possible to detect a significant change in the expression of ERp57 protein.

Figure 3.6. ERp57 expression is not silenced by any of the three dsRNA sequences tested.

NIH3T3 cells at 20% confluence in a 6-well plate were transfected with 2µl of T7 dsRNA specific for ERp57 or GFP and 5µl lipofectamine. 48 hours later expression of ERp57 and the housekeeping gene calnexin was assessed by western blot.



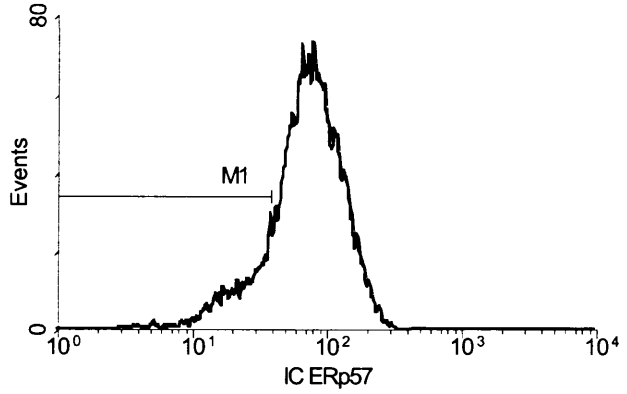
ERp57 levels were also measured by intracellular FACS. It can be seen that the expression of ERp57 as measured by intracellular FACS, is not normally distributed after treatment with either RNA I or RNA II (Fig. 3.7 a and b). The presence of a small, low expressing population of cells in figure 3.7b, suggests some suppression of ERp57 protein levels in these cells. However, these results were not consistent, and when the data are overlaid with the irrelevant dsRNA the suppression is cast into doubt (Fig. 3.7c). The expression of ERp57 in cells transfected with irrelevant dsRNA is normally distributed, which shows that the population of low expressing cells in figure 3.7b is not purely an artefact of the intracellular staining. However, the peak of ERp57 expression in these cells lies between the high and the low expressing cells transfected with RNA I or RNA II.

Suppression of ERp57 by transfection of dsRNA II, produces at best a 75% reduction in ERp57 expression in 30% of the population (Fig. 3.7b – M1), which is consistent with the transfection efficiency of 30-40%. However, unlike GFP expression, which is suppressed to below detectable levels by dsRNA, expression of ERp57 remains significant even in the putative population of RNAi suppressed cells.

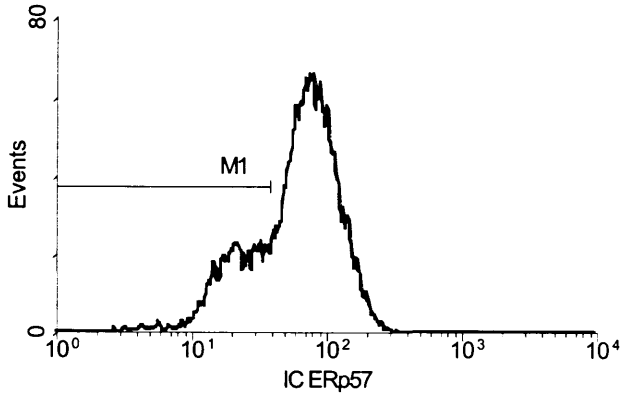
Figure 3.7. DsRNA sequences I and II may cause low level suppression of ERp57.

NIH3T3 cells at 20% confluence in a 6-well plate were transfected with 2µl of T7 dsRNA and 5µl lipofectamine. 48 hours later expression of ERp57 was assessed by intracellular FACS. a) RNA sequence II - target ERp57, b) RNA sequence I - target ERp57, c) RNA sequence I (black), RNA sequence III - target GFP (grey).

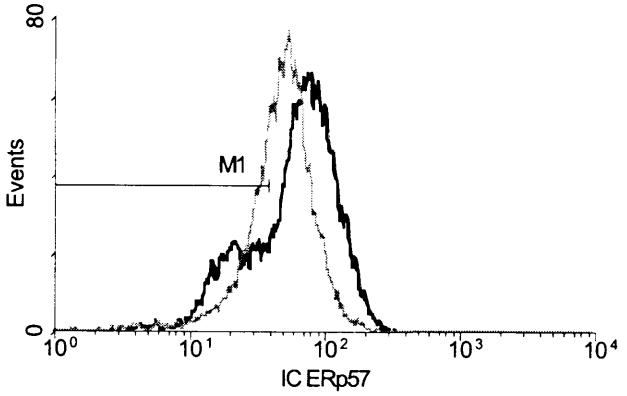
a)



b)



c)



3.4 Discussion

Although this level of suppression may be enough to affect the function of some proteins it was felt that it was unlikely to be sufficient for a high expressing housekeeping gene like ERp57. Indeed analysis of cell surface expression of mouse class I in dsRNA treated cells showed no change in expression (data not shown). Other researchers have also commented that after 90% suppression of ERp57 there is no change in phenotype with respect to cell surface class I and peptide loading (Williams, 2006) and that ERp57 can still be found in associated with the PLC (Cresswell personal communication).

It would have been possible to try and stably transduce cells to express dsRNAs against ERp57 or to use RNAi libraries to suppress ERp57. However, RNAi library technology is expensive and since the results of this preliminary work were not promising and the fact that other eminent researchers had also found the approach of RNAi unsuccessful for ERp57 it was decided not to pursue this further.

Since this work was undertaken, mice have been created with ERp57 knockout in the B cell compartment (Garbi et al., 2006). 2 loxP sites were directed to flank exons 2 and 3 of the ERp57 gene and 'floxed' mice were produced *Pdia*$^{fl/fl}$. When Crossed with Cre deleter mice no homozygous ERp57 negative offspring were obtained in the F2 progeny, showing that ERp57 is essential for *in utero* development. However, using tissue specific Cd19-Cre mice it was possible to produce mice with ERp57 knockout in the B cell compartment. ERp57 deficient fibroblasts were also produced by *in vitro* recombination of SV40 T antigen immortalised *Pdia*$^{fl/fl}$ fibroblasts. Characterisation of both ERp57 deficient B cells and the fibroblast cell line showed that there is a 50% reduction in the expression of K^b in these cells by both western blot and by flow cytometry. This phenotype is explained by poor incorporation of heavy chain into the peptide-loading complex and rapid trafficking of K^b molecules resulting in instability and reduced half life at the cell surface. A smaller effect is also seen on the expression of D^b. Most interestingly the absence of ERp57 leads to the loss of almost all heavy chain from both TAP and tapasin immunoprecipitates. Given this fact it is surprising that the phenotype is so mild, since incorporation into the peptide-loading complex (Tan et al., 2002) and association with tapasin (Williams et al., 2002) has previously been shown to be very important for peptide loading. However, ERp57 is clearly important for stabilising the interaction of heavy chain with the peptide-loading complex and it may be that like dependence on tapasin, the extent to which ERp57 is needed for class I

loading is allele specific. Previously it had been thought that tapasin was sufficient to form a bridge between TAP and heavy chain but this work shows that ERp57 is also important for this interaction. ERp57 may strengthen the interaction between heavy chain and tapasin either by disulfide bonding with tapasin, altering its conformation and allowing it to bind heavy chain or ERp57 may bind to both heavy chain and tapasin thereby stabilising the interaction between the two. Whether the only requirement for ERp57 is in the stabilisation of the peptide-loading complex in unclear and a full discussion of the other possible roles of ERp57 is made in chapter 7. Briefly, since ERp57 is a member of the protein disulfide isomerase family it is thought likely that its function in the peptide-loading complex may be to ensure that the disulfide bond in the α_2 domain of the class I heavy chain remains oxidised. By transfection of a mutant tapasin, which fails to associate with ERp57, into tapasin deficient cells it has been shown that ERp57 does have a role in the oxidation of HLA-B44 (Dick et al., 2002). However, in ERp57 knockout cells no difference was detected in the redox state of MHC class I molecules isolated from the peptide-loading complex (Garbi et al., 2006).

4 Expression of linked chain MHC class I constructs as a tool for dissecting the assembly pathway

4.1 Introduction

As discussed in section 1.3 there is now a large body of knowledge about the molecular processes behind successful MHC class I antigen presentation (Villadangos et al., 2005;Flutter and Gao, 2004). Antigenic peptides are mainly produced by proteasome by the turnover of cytosolic proteins and the breakdown of DRiPs (Defective Ribosomal Proteins) (Reits et al., 2000;Khan et al., 2001). TAP then transports peptides from the cytosol to the ER (Trowsdale et al., 1990;Deverson et al., 1990;Spies et al., 1990) (Anderson et al., 1991), where they are trimmed to size by the aminopeptidase ERAAP (Serwold et al., 2002;Saric et al., 2002;York et al., 2002). Meanwhile, newly synthesised MHC class I heavy chain molecules associate with calnexin in the 'early complex' of class I folding (Degen and Williams, 1991;David et al., 1993). Subsequently heavy chain pairs with β_2m and calnexin is dropped in favour of calreticulin (Farmery et al., 2000;Sadasivan et al., 1996). The class I β_2m-HC heterodimer and antigenic peptide are thought to come together in the peptide-loading complex, and failure of class I molecules to associate with the peptide-loading complex generally leads to poor antigen presentation (Lewis et al., 1996;Ortmann et al., 1997).

However, there are a number of unanswered questions surrounding the details of class I assembly and peptide loading. It is clear that calnexin, calreticulin, tapasin and ERp57 all associate with class I molecules during the process of class I folding (Farmery et al., 2000;Sadasivan et al., 1996). However, the role of each of these molecules in antigen processing is not well characterised. The mechanisms of β_2m recruitment to heavy chain, peptide loading and release of loaded class I molecules from the peptide-loading complex also remain unclear.

In order to investigate the folding mechanisms of MHC class I molecules, fusion proteins have been constructed, creating class I molecules, which after synthesis are effectively partially assembled. Fully folded MHC class I molecules consist of three different components - heavy chain, β_2m and antigenic peptide. In total five different constructs, containing one or more of these three components have been made. Each of the constructs contains β_2m and they are designed as follows (information repeated from section 1.4 for the reader's convenience):

β_2m: (human) 'β_2m'.

PB: 'peptide - linker 1 - β_2m'.

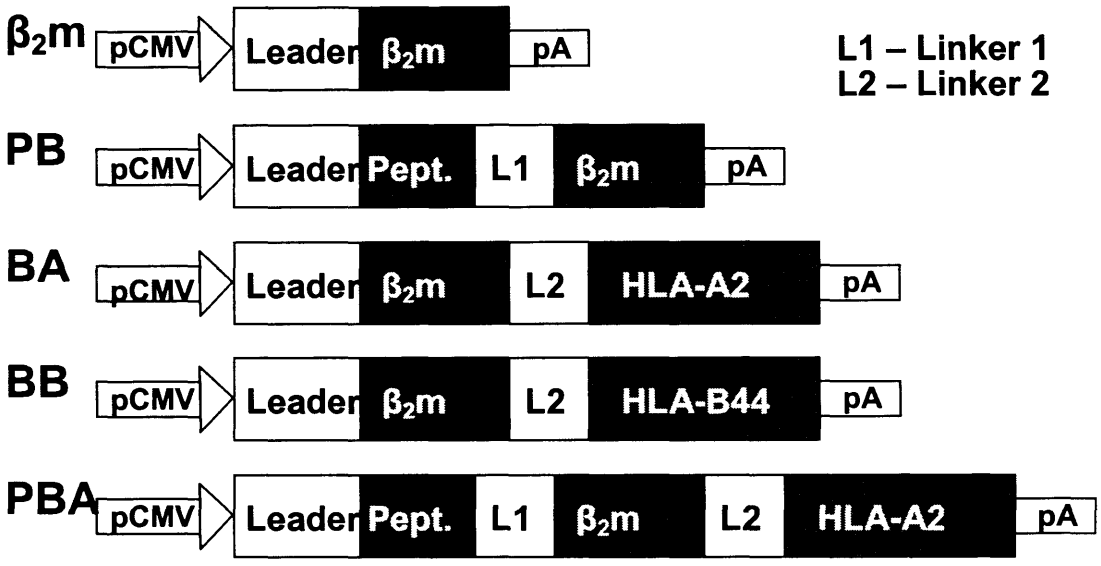
BA: 'β_2m - linker 2 - HLA-A*0201'.

BB: 'β_2m'-'linker 2'-'HLA-B*4402'.

PBA: 'peptide - linker 1 - β_2m - linker 2 - HLA-A*0201'.

Where 'linker 1' codes for GGGGGGSGGSGGSGG, 'linker 2' codes for GGGGSGGGGSGGGGS and 'peptide' codes for the HLA-A2 binding peptide GILGFVFTL derived from influenza virus matrix protein (Fig. 4.1). The BA construct was provided by Prof. W Bodmer (Oxford University, UK). Dr. Bin Gao (UCL, UK) created the fusion constructs PB and PBA, and engineered the BB construct from BA by replacing HLA-A2 with HLA-B*4402 (provided by Dr. C Peh. Oxford University, UK).

Figure 4.1. Schematic representation of MHC class I fusion proteins. *(see also Figure 1.4b)*



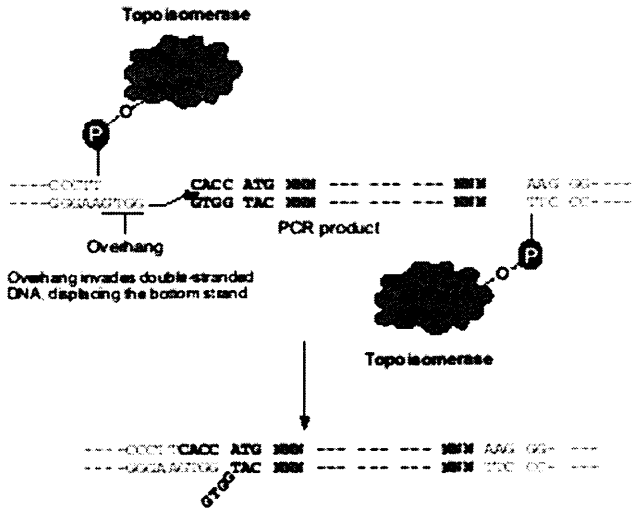
Use of these partially assembled class I molecules provides a new approach to studying the different mechanisms of class I antigen processing. By expressing fusion proteins in different deficient cell lines, and by examining their intermolecular interactions with other proteins and their rate of trafficking, it is possible to address issues such as: the role of calreticulin in β_2m recruitment and the importance of peptide binding on the release of class I molecules from the peptide loading complex. These ideas are

discussed further in sections 5.1 and 6.1. In order to validate the use of fusion constructs in the study of class I folding, they were first assessed for their ability to express at the cell surface and present antigen to T cells. In the work presented in this chapter, β_2m, BB and PB have been cloned into a lentiviral vector pLenti6-V5-D-TOPO (Invitrogen) and high titre virus produced. Lentivirus was used to express β_2m in the human β_2m deficient colon carcinoma cell line HCT (Dexter et al., 1979) and Dr. Bin Gao (UCL, UK) expressed the fusion proteins PB, BA and PBA in HCT cells. Since HCT cells express no cell surface class I, it is easy to assess the ability of the introduced proteins to restore cell surface expression of class I and to present antigen.
The advantage of using a viral system to stably express MHC class I constructs, is that the viruses can be used to infect several cell lines and this is useful in the study of different chaperones involved in class I antigen presentation.

4.1.1 Virapower lentivirus expression system (Invitrogen)

The Virapower lentivirus expression system allows production of a replication-incompetent HIV-based virus, capable of stably transducing most mammalian cells and allowing expression of the incorporated gene of interest. Virus is produced in the HEK 293FT (fast growing 293 cells expressing SV40 large T antigen) cell line by simultaneous transfection of four plasmids encoding different vital components of the lentivirus.
The first plasmid pLenti-6-V5 is the pLenti-based expression vector into which the gene of interest is cloned (Appendix I). The gene of interest is amplified by blunt ended PCR and inserted into the plasmid by TOPO cloning. The forward primer is designed with a leader sequence CACC, which allows directional cloning (see Fig. 4.2) and the reverse primer may be designed such that there is no STOP codon at the end of the gene product causing the protein product to be tagged by a V5 epitope. The plasmid is supplied in an 'open' form which allows the gene to be cloned directly in front of the CMV promoter. An overhang complementary to the CACC forward primer leader sequence allows directional cloning catalysed by topoisomerase enzymes attached to each end of the open plasmid (Fig. 4.2).

Figure 4.2. The Topoisomerase reaction.



(Taken from Invitrogen instruction manual 'pLenti6/V5 Directional TOPO® Cloning Kit' Version C. With kind permission from Invitrogen Eurotech.)

The pLenti vector contains modified 5' and 3' LTRs (Dull et al., 1998) and the ψ packaging signal (Luciw, 1996), to allow packaging into virions, and also genes required for effective expression of the protein in mammalian cell lines such as the Rev Responsive element. The plasmid also contains a blasticidin resistance gene to allow selection of transduced cells, an ampicillin resistance gene for selection in E. coli and the pUC origin for high-copy replication.

The 3 other plasmids contain the genes required for production of virus in the packaging cell line. By separating the genes onto separate plasmids the safety of the virus system is greatly increased. The lack of these genes in the pLenti vector also makes the produced virus replication incompetent for infection of mammalian cells.

The vital genes expressed by the other plasmids include:

pLP1: Gag/pol fusion protein.

pLP2: HIV-1 Rev ORF, LTR polyadenylation signal.

pLP/VSV G glycoprotein, Human β-globin intron and polyadenylation signal.

(See Appendix I for plasmid maps.)

Viruses produced in 293FT cells are harvested from the supernatants and can theoretically be used to transduce any mammalian cell lines. A positive control virus expressing β-galactosidase (β-Gal), which can be easily detected by cell staining, is used to verify that the viral system is working. Cells can either be transduced to efficiently deliver transient protein expression to target cells or can be used to produce stable cell lines by selecting for transduced cells using the antibiotic blasticidin which is

a protein synthesis inhibitor (TAKEUCHI et al., 1958). The blasticidin resistance gene contained within the pLenti6 vector codes for a blasticidin deaminase enzyme (*bsd*), which renders the blasticidin harmless to transduced cells (Kimura et al., 1994).
The lentivirus system developed in this chapter has several useful characteristics. The envelope of this virus expresses VSV-G (the glycoprotein of vesicular stomatosis virus), which means it is able to transduce with high efficiency a wide range of mammalian cells (Emi et al., 1991). Since some of the cells lines available with deficiencies in class I or class I antigen processing are of murine origin and others are of human origin, this is useful, because the same virus can be used transduce all of these cell lines. The CMV promoter drives powerful expression of the introduced genes in mammalian cells (Boshart et al., 1985) and because the plasmid integrates into the genome of the host cell this expression can be long lasting. The blasticidin resistance gene allows selection of transduced cells and facilitates production of stable cell lines where necessary. Unlike some viral systems lentivirus is also largely non-toxic to the cells and since the titre of most preparations is about 10^6 virions/ml, it can also be used to transduce whole cell populations for transient expression.

4.1.2 Cell cytotoxicity assay

In order to test the ability of class I fusion proteins to present antigen to T cells a cell cytotoxicity assay was used. Because T cells can be difficult to grow and maintain, the assay was set up and optimised using a Natural Killer (NK) cell line NK92.
NK cells are a heterogenous lymphoid population capable of killing a wide variety of target cells that have been transformed or infected with virus. Target cell killing often negatively correlates with the expression of MHC class I and this led to the 'missing self' hypothesis of NK cell recognition (Ljunggren and Karre, 1990). The decision to kill is determined by a balance between activating and inhibitory signals received by the NK cell. KIRs (Killer Inhibitory Receptors) and ILTs (Immunoglobulin Like Transcripts) mainly interact with either classical or non-classical MHC class I molecules and are important in the control of killing. KIRs differ in their reactivity to a range of class I and can either promote or inhibit NK killing upon interaction with the appropriate class I. KIRs containing an ITIM (Immunoreceptor Tyrosine-based Inhibition Motif) sequence in their cytoplasmic tail inhibit killing, whereas KIRs that do not contain ITIMs promote killing (Moretta and Moretta, 1997). A number of other activating and inhibitory receptors also play a role in the control of target cell lysis.

NK92 cells are a human cell line with a phenotype similar to activated NK cells and capable of killing the chronic myelogenous leukaemia cell line K562 (Gong et al., 1994). The killing activity of NK92 cells is generally acknowledged to be high and most tumour cells, including those displaying class I molecules are susceptible to lysis (Komatsu and Kajiwara, 1998). K562 cells (Lozzio and Lozzio, 1975) are known to be sensitive to NK killing and have been used to determine the relative natural cytotoxicity of human lymphocytes (West et al., 1977). Conversely the CEM.NKR cells were generated by screening CEM cells for survival of NK killing (Howell et al., 1985). CEM.NKR cells are calnexin deficient and it is hypothesised that this deficiency leads to a failure of trafficking of cells surface markers that normally activate NK cell killing (Malyguine et al., 1998).

HCT cells have not been characterised for their susceptibility to NK92 killing. The use of K562, NKR and HCT cell lines should allow an assessment of the susceptibility of HCT cells to NK92 cell killing. A comparison between killing of HCT cells and HCT cells expressing class I fusion proteins will indicate the importance of class I expression in the susceptibility of a target cell to killing by NK92 cells.

4.1.3 Objectives

In this chapter, class I fusion proteins will be cloned into a lentivirus and viruses will be produced, which may be used to transduce any mammalian cells.

Using prepared lentivirus the linked chain construct β_2m-B44 (BB) will be introduced into murine K41 cells and the calreticulin deficient cell line K42 and cell lines stably expressing β_2m-B44 will be established.

Stable expression of different class I fusion proteins will also be established in the β_2m deficient human HCT cell line. Together the resulting cell lines, HCT, HCT β_2m, HCT PB (peptide linked β_2m), HCT BA (β_2m linked to HLA-A2) and HCT PBA (peptide linked to β_2m and HLA-A2) provide a panel of cell lines each expressing class I molecules at different stages of assembly. It is hypothesised that each of the constructs will recover cell surface expression of MHC class I in HCT cells. Additionally, the peptide of the PB and PBA constructs will allow HCT cells expressing these constructs to stimulate the GILGFVFTL (influenza matrix protein derived peptide) specific T cell clone CTL_{FLU}. HCT β_2m and and HCT BA cells will be also be able to stimulate

CTL_{FLU}, when provided with either flu peptide or when endogenously expressing whole flu matrix protein.

The effect that class I expression has on the susceptibility of HCT cells to NK92 cell killing will also be assessed.

4.2 Methods

4.2.1 Lentivirus production

4.2.1.1 TOPO cloning

The gene sequences to be incorporated into lentiviral constructs (see table 4.2) were amplified using blunt ended PCR with the DNA Polymerase PfU (Stratagene). PCR was performed as laid out in section 2.4.4 using the primers in table 4.1.

Table 4.1. Primers for PCR

Primer	Sequence	Tm (°C)	Features
B44 F TOPO	CACCATGCGGGT CACGGCGC	56	Start B44. CACC leader
B44 R	AGCTGTGAGAGA CACATCAG	58	End of B44 without STOP codon
T7 TOPO (T7- RNA Polymerase promoter site)	CACCTAATACGA CTCACTATAGG	52	CACC leader
BGH (Bovine Growth Hormone polyadenylation site)	TAGAAGGCACAG TCGAGG	52.5	Common reverse primer site.
M13 TOPO (M13 – Common primer site from the N-terminus of β-Galactosidase gene)	CACCGTAAAACG ACGGCCAGT	44	CACC Leader

Table 4.2. Characteristics of Lentiviral constructs

Construct	Description	Primers used to amplify gene
hβ_2m	Human β_2m	T7TOPO, BGH. From pcDNA3.1-β_2m.
B44-V5	HLA-B44 with V5 epitope tag.	B44 F TOPO, B44 R. From pUC13-B*4402.
β_2m-B44-V5	As β_2m-B44 with a V5 epitope tag	T7TOPO, B44R. From pcDNA3.1-β_2m-B44.
Lac Z	β-galactosidase gene expressed	Provided with TOPO vector (Invitrogen)

PCR products were run on a 1% agarose gel and purified before use in the TOPO cloning reaction as below:

1µl pLenti TOPO vector (Invitrogen)

4µl gel purified PCR product

1µl salt solution (1.2M NaCl, 0.06M $MgCl_2$)

The reaction was gently mixed and allowed to proceed for 5-10 mins at room temperature.

4.2.1.2 Transformation and DNA production

2µl of product from the TOPO cloning reaction was used to transform TOP10 competent *E.coli* (Invitrogen). Transformation products were plated onto LB agar plates with 50µg/ml blasticidin and 50µg/ml ampicillin and incubated for 12-18 hours at 37°C. 2-5ml LB broth with 50µg/ml ampicillin was inoculated with a single colony from the plate and incubated in a 37°C shaker overnight. Bacterial broth was then used to miniprep the plasmid and DNA was digested with restriction enzymes to confirm the presence of the inserted gene. Once analysed a successfully cloned plasmid was amplified by Qiagen Maxiprep. The plasmid product was quantified by spectrophotometry and sent to the Wolfson Institute of Biomedical Research (WIBR) at University College London for sequencing.

4.2.1.3 Virus Production

The day before transfection, 293FT cells were trypsinised and counted, and 5 x 10^6 cells plated on a 10cm diameter plate in 10ml of normal medium (see Table 2.1). On the day of transfection, medium was removed and the cells washed with PBS. 5ml Opti-MEM® I medium was added to the cells. 3µg of pLenti expression vector, and 9µl of the optimised packaging mix (Invitrogen) containing pLP1, pLP2 and pLP/VSVG (see section 4.1.1) were diluted in 1.5ml of Opti-MEM® I medium. Lipofectamine 2000 was mixed gently and 36µl was diluted in 1.5ml of Opti-MEM® I medium. After 5 minutes the diluted DNA was mixed with the diluted Lipofectamine and the mixture was incubated for 20 minutes at room temperature.

The DNA and lipofectamine mixture was then added dropwise to the 293FT cells and the plate was rocked gently before returning to the incubator. After overnight incubation the medium was gently removed and the cells washed in PBS. 10ml of normal culture medium was added, and the cells were incubated for a further 48 hours. Following

incubation supernatant containing produced virus was removed and centrifuged at 300g for five minutes to remove cell debris.
Virus containing medium was then dispensed into cryovials in 1 ml aliquots and stored at - 80°C. 'Freeze – thawing' of virus was avoided as much as possible and was performed a maximum of three times as it can reduce the efficacy of viral infection.

4.2.1.4 Determining blasticidin sensitivity

Since transduced cells are to be selected using blasticidin it is necessary to determine the drug sensitivity of the cell line to be transduced.
Cells were plated at around 25% confluence on a 6-well plate. Medium at various concentrations of blasticidin from 0 – 10μg/ml was added to the cells. Fresh blasticidin containing medium was added to the cells every 2-3 days or as necessary. Cells were grown for up to ten days, or until there were no surviving cells.
The lowest concentration of blasticidin at which no cells survived after 10 days of culture was subsequently used to select transduced cells.

4.2.1.5 Titering lentiviral Stock

Virus can be titred using any mammalian cell line. The human fibrosarcoma HT1080 cells are most easily transduced and generally give a titre about 10-fold greater than NIH 3T3 cells, which were used here. (Invitrogen, 2005)
0.005 – 1μl of virus stock was used to infect 25% confluent NIH 3T3 cells in a six well plate. After 10 days of drug selection medium was removed and cells were washed with PBS and then stained with 1ml 1% crystal violet, which stains live cells, in 10% ethanol for 10 mins. Crystal violet was removed and cells washed twice in PBS and stained colonies were counted.

4.2.2 Stable transduction of mammalian cell lines

4.2.2.1 Cell transduction

Cells were plated in 6-well plates at about 25% confluence (~ 200,000 cells) 1 day prior to transduction. On day of transduction virus was thawed and 0.5ml fresh medium containing polybrene at a concentration of 12μg/ml (final concentration after 1 in 2 dilution was 6μg/ml) was added to each well of the 6-well plate.
Viral stock was diluted into medium at various concentrations (Multiplicity of Infection, (MOI) from 0.001 - 2) and 500μl was added to each well of the 6-well plate. After 24 hours, medium was removed and replaced with fresh medium. After a further 24 hours, medium was removed and fresh medium containing blasticidin was added. Drug selection was carried out for at least 10 days and colonies of transduced drug resistant cells formed.

4.2.2.2 Expansion of virally transduced clones

During blasticidin treatment distinct cell colonies formed from each transduced cell. 8 colonies were selected with an inoculation loop and transferred to separate wells of a 24 well plate containing normal medium (see Table 2.1) supplemented with blasticidin. Clones were subsequently screened for expression of protein by flow cytometry or western blot.

4.2.2.3 Expression of β-galacosidase

Expression of β-galactosidase in Lac Z transduced cells was assessed by the breakdown of X-gal substrate.
Stock ferrous solution - 5mM KFerricyanide, 5mM KFerrocyanide, 2mM $MgCl_2$ was made up in PBS and stored at 4°C.
Cells were washed x2 in cold PBS, fixed for 5mins in 4% paraformaldehyde and washed again x2 in cold PBS. X-Gal (5-bromo-4-chloro-3indoly-β-D-galactopyranoside) was diluted 1 in 20 into the stock ferrous solution and added dropwise to cells to cover. Cells were incubated at 37°C for at up to 16 hours or until blue staining appeared.

4.3 Results

4.3.1 Production of lentivirus to express class I constructs

4.3.1.1 Molecular cloning

Genes were successfully amplified from plasmid DNA as outlined in Table 4.2 by PCR. Amplification products were run on 1% Agarose gels and stained with EtBr. Amplification products were used for TOPO cloning, and miniprep DNA from the resulting colonies was analysed by restriction digestion to release the cloned insert. In each case the size of the bands for the PCR product and the released insert of the chosen clones were verified.

The β_2m gene is 360bp and distance between the primer sites in the original pcDNA3.1 vector is 181bp. The PCR amplification product should therefore be 541bp long. PCR yield was poorer with the T7 TOPO primer, which incorporates the CACC leader sequence to allow directional cloning, than with the normal T7 primer and a weaker second band of larger molecular weight is seen. However, the smaller PCR product is of the correct size (Fig. 4.3) and the yield obtained with the T7 TOPO primer in conjunction with BGH primer was sufficient to successfully clone the gene into the TOPO vector (Fig. 4.4).

Figure 4.3. PCR amplification from pcDNA 3.1 – β_2m yields band of 541bp.
β_2m was amplified from pcDNA 3.1 - β_2m plasmid with BGH reverse primer and either T7 or T7 TOPO primer, by 30 rounds of PCR using PfU DNA Polymerase. Denaturing, 30 secs at 94°C. Annealing, 60 secs at indicated temperature. Polymerisation, 60 secs at 72°C.

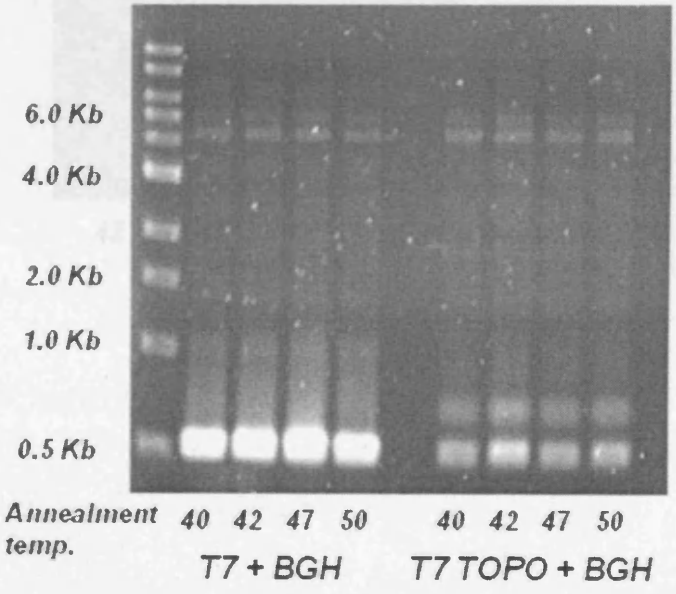
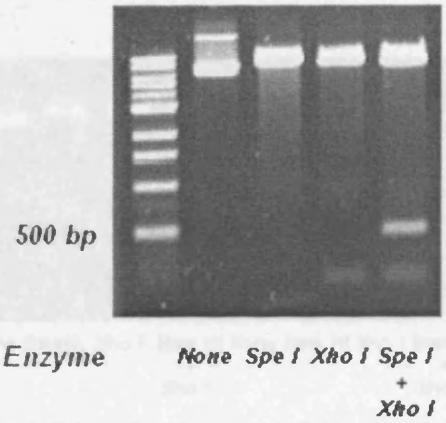


Figure 4.4. pLenti 6-β_2m contains inserted gene. *Miniprep DNA from a colony formed after β_2m TOPO cloning was cut with the indicated restriction enzymes to linearise the plasmid or to release the cloned gene.*



The B44 gene was amplified using B44F TOPO and B44R primers (table 4.1) yielding a product consistent with the predicted size of 1086bp (Fig. 4.5). The PCR amplification product was used for TOPO cloning and resulting pL-B44 plasmids were digested to confirm incorporation of the gene (Fig. 4.6).

Figure 4.5. PCR amplification from pUC13 – B*4402 yields a product of 1086bp. *HLA-B*4402 was amplified from pUC13 – B*4402 with B44F TOPO forward primer and B44R reverse primer by 30 rounds of PCR using PfU DNA polymerase. Denaturing 30 secs at 94°C. Annealing 60 secs at indicated temperature. Polymerisation 60 secs at 72°C.*

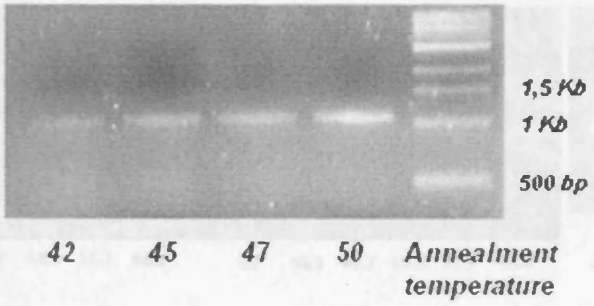
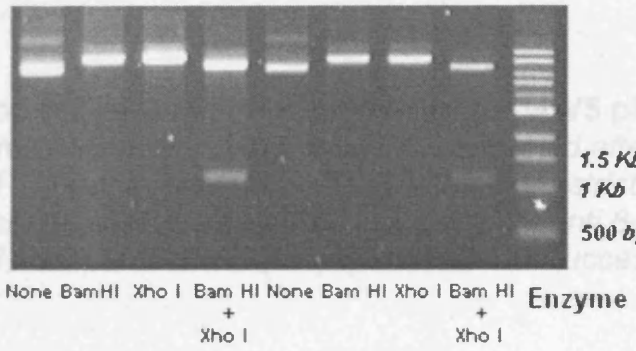
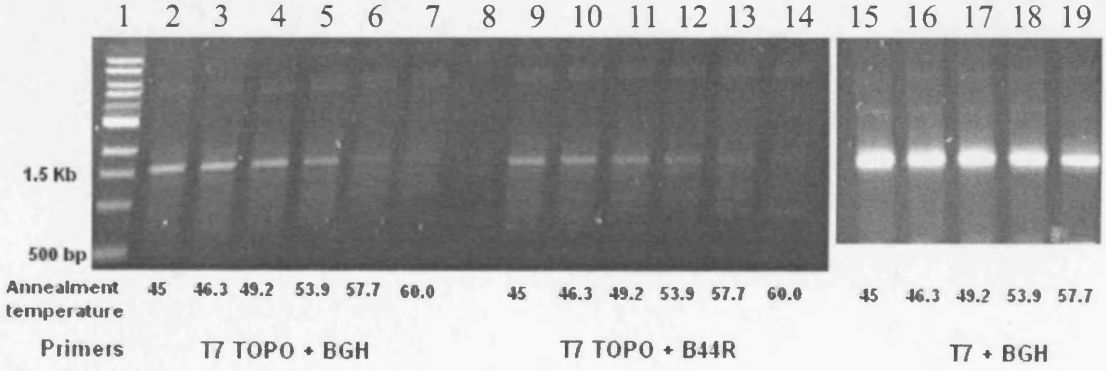


Figure 4.6. pLenti 6-B44 contains inserted gene. *Miniprep DNA from a colony formed after B44 TOPO cloning was cut with the indicated restriction enzymes to linearise the plasmid or to release the cloned gene.*



The linked chain construct β_2m-B44 (BB) was amplified for TOPO cloning using the T7TOPO forward primer and the B44R reverse primer. The gene for β_2m-B44 is 1419bp. Amplification yielded a product of the appropriate size and was used for TOPO cloning (Fig. 4.7). Resulting pLenti - β_2m-B44, when cut with Spe I and Xho I also released a band of the expected size (Fig. 4.8).

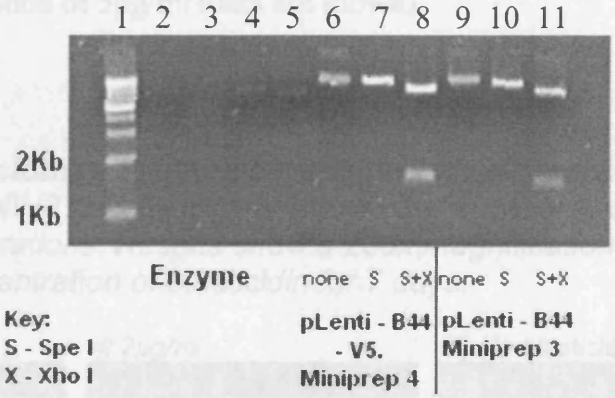
Figure 4.7. β_2m-B44 (BB) was amplified by PCR from pcDNA 3.0 - β2m-B44. *β_2m-B44 was amplified from pcDNA 3.0 - β_2m-B44 with the indicated primers by 30 rounds of PCR using PfU DNA Polymerase. Denaturing, 30 secs at 94°C. Annealing, 60 secs at indicated temperature. Polymerisation, 90 secs at 72°C.*



Lanes 2-7: PCR product from the T7 TOPO, BGH primer pair.
Lanes 9-14: PCR product from the T7 TOPO, B44R primer pair used to make 'pLenti β2m -B44-V5'.
Lanes 15-19: PCR product from the T7, BGH primer pair.

PCR reaction performed with the T7 TOPO primer was again less efficient than with the T7 primer but the products obtained with T7 TOPO and B44R primers at an annealing temperature of 45°C were sufficient for the purposes of TOPO cloning (Fig. 4.7).

Figure 4.8. pLenti 6-β_2m-B44 and pLenti 6- β_2m-B44-V5 plasmids contain the inserted genes. *Miniprep DNA from colonies formed after β_2m-B44 and β_2m-B44-V5 TOPO cloning was cut with the indicated restriction enzymes to linearise the plasmid or to release the cloned gene. 'pLenti β_2m-B44' (Lanes 9-11) and 'pLenti β_2m-B44-V5' Lane (6-8). (Lanes 2-5 unsuccessful cloning product).*

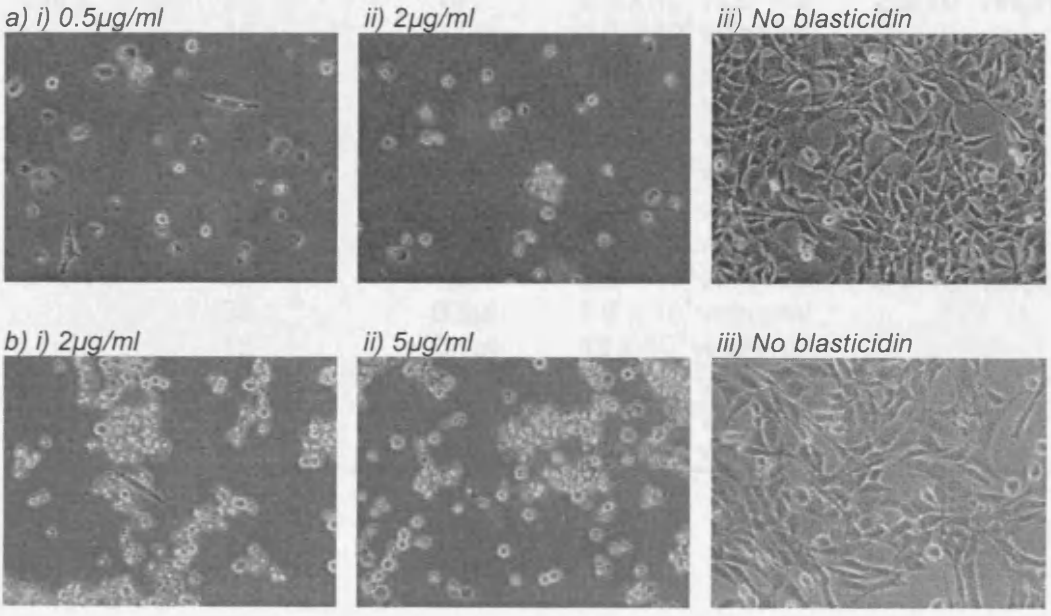


4.3.1.2 Blasticidin resistance

To establish the blasticidin sensitivity of cell lines for subsequent selection of transduced cells, a range of blasticidin concentrations was tested. K41 cells treated with 10μg/ml blasticidin were killed within 2 days. Blasticidin at 2μg/ml killed K41 cells effectively after 10 days, however when treated with 0.5μg/ml blasticidin some cells survived for more than 10 days (Fig. 4.9a).

NIH3T3 cells were a little more resistant to blasticidin, with a few live cells still visible after 10 days of treatment with blasticidin at 2μg/ml. However, at 5μg/ml cells were all killed within 10 days of treatment (Fig. 4.9b). HCT cells were also killed effectively at a blasticidin concentration of 5μg/ml (data not shown).

Figure 4.9. Blasticidin resistance determined for different cell lines.
K41 cells (a) and NIH3T3 cells (b) were tested for survival at a range of blasticidin concentrations. Results show a 200x magnification of cells grown in the indicated concentration of blasticidin for 7 days.



4.3.1.3 Infectivity of virus and Lac Z expression in transduced cells

The infectivity of the produced virus was assessed by the transduction of NIH3T3 cells. These cells transduce relatively easily, although the titre is around 10-fold lower than that typically obtained in HT1080 cells (Invitrogen, 2005). After 10 days of selection with blasticidin at 5μg/ml, cells that have not been transduced will have been killed. The colonies formed correspond to live cells transduced with virus encoding the blasticidin resistance gene. These cells were stained with crystal violet. Estimated titres in HT1080 of around $10^5 - 10^6$ virion/ml were obtained for each virus produced (Table 4.3)

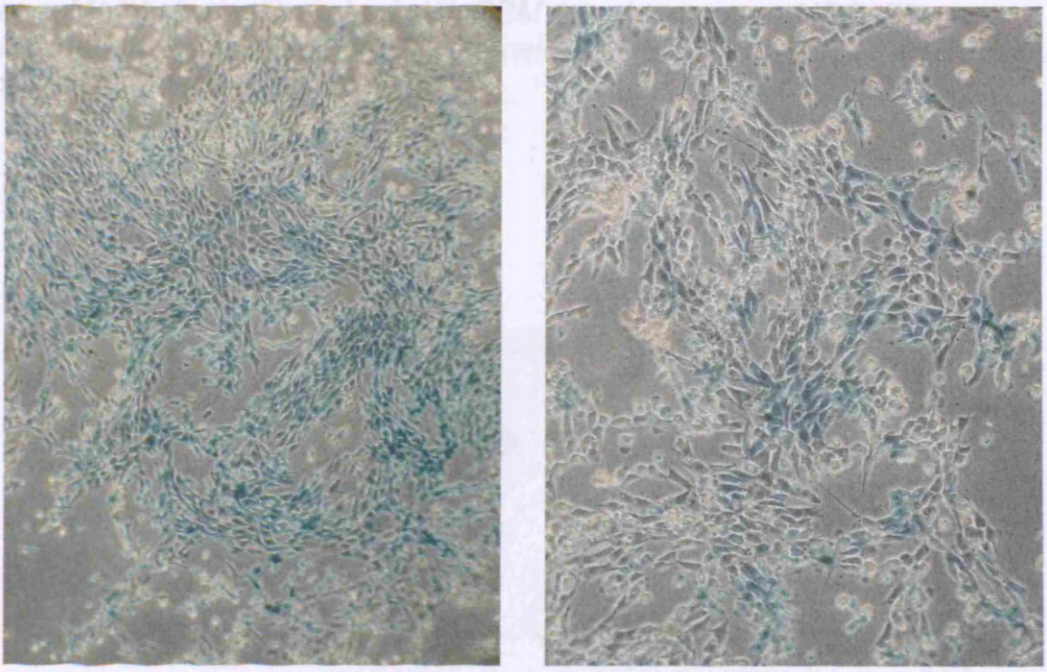
Table 4.3. Titre of lentivirus preparations. *Lentivirus preparations were used to transduce NIH3T3 cells. Cells were selected for 10 days with for 5μg/ml blasticidin and surviving cell colonies stained with crystal blue.*

Virus	Crystal violet colonies	Volume of virus	Titre in NIH3T3 cells	Estimated titre in HT1080
Lac Z	24	1μl	2.4×10^4 virion/ml	2×10^5 virion/ml
	10	0.5μl	2.0×10^4 virion/ml	
	2	0.1μl	2.0×10^4 virion/ml	
β_2m	45	10μl	4.5×10^3 virion/ml	9×10^4 virion/ml
	36	2μl	1.8×10^4 virion/ml	
	10	1μl	1.0×10^4 virion/ml	
B_2m-B44	100	0.5μl	2.0×10^5 virion/ml	10^6 virion/ml
	10	0.1μl	1.0×10^5 virion/ml	
B44-V5	83	1μl	8.3×10^4 virion/ml	8×10^5 virion/ml
	38	0.5μl	7.6×10^4 virion/ml	
	13	0.1μl	13×10^4 virion/ml	
β_2m-B44-V5	60	2μl	3.0×10^4 virion/ml	3×10^5 virion/ml
	31	1μl	3.1×10^4 virion/ml	
	17	0.5μl	3.4×10^4 virion/ml	

Expression of β-galactosidase was assessed in NIH3T3 cells transduced with Lac Z virus (Fig. 4.10a). After blasticidin selection about half of the surviving colonies expressed β-gal. Most of these colonies were homogeneous, such that either all or none of the cells within the colony expressed β-gal. In some regions it appeared that colonies were mixed (Fig. 4.10b).

Figure 4.10. Lac Z expression in NIH3T3 cells.
NIH 3T3 cells were transduced with Lac Z virus and selected for 10 days with blasticidin. Resulting colonies were stained for β-gal expression and viewed by microscopy. a) Lac Z expressing colony (x100) b) Mixed expression colony (x200).

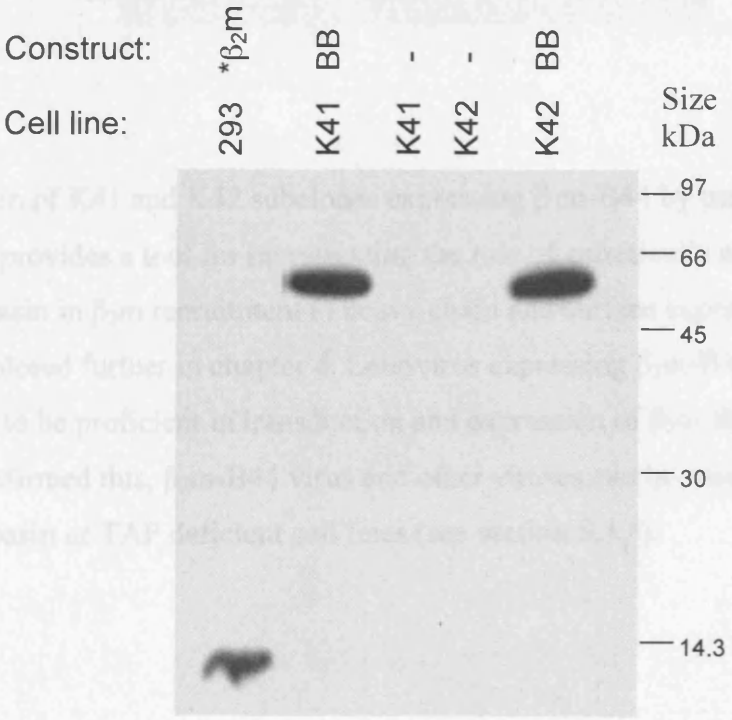
a) *b)*



4.3.2 Expression of β_2m-B44-V5 in K41 and K42 cells.

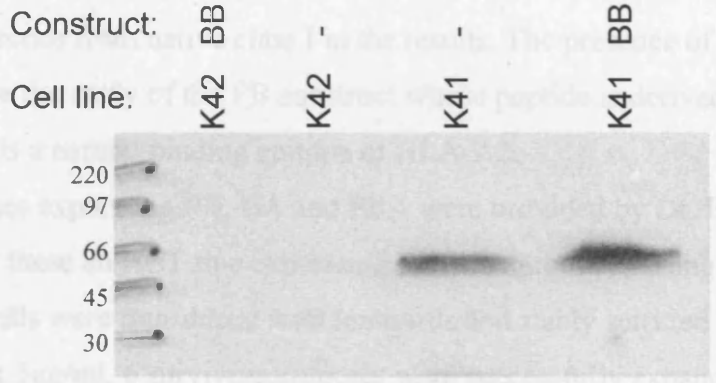
Murine K41 fibroblast cells and calreticulin deficient K42 cells (Mesaeli et al., 1999) were transduced with β_2m-B44-V5 (BB) virus and treated with blasticidin. Clones were assessed for production of β_2m-B44-V5 protein by western blot using BBM.1 antibody specific for human β_2m (Fig. 4.11). Positive clones were obtained in both K41 cells and K42 cells transduced with virus; as expected no protein was detected in normal K41 and K42 cells, since these are murine cells and BBM.1 does not bind murine β_2m.

Figure 4.11. β_2m-B44 is expressed in transduced K41 and K42 cells.
*K41 and K42 cells were transduced with lentivirus expressing β_2m-B44-V5, cells were selected by addition of blasticidin and clones screened for expression of protein by western blot. Samples were loaded at 2 x 10^5 cells per lane. Membrane was probed with BBM.1 and α-mouse - HRP. Film was exposed for 1 minute. * The human cell line HEK293 was used as a positive control expressing human β_2m.*



A western blot for calreticulin was also performed on these cells. K41 cells and all K41 clones transduced with β_2m-B44-V5 were confirmed as calreticulin positive, but K42 cells and K42 β_2m-B44-V5 clone 5 were confirmed as calreticulin deficient (Fig. 4.12).

Figure 4.12. Calreticulin is expressed in K41 but not K42 cells.
Samples of 4 x 10^5 cells were separated by SDS PAGE and analysed by Western Blot using α-calreticulin rabbit serum and α-rabbit - HRP. Film was exposed for 1 minute.



The creation of K41 and K42 subclones expressing β_2m-B44 by transduction with lentivirus, provides a tool for investigating the role of calreticulin and possibly also of human tapasin in β_2m recruitment to heavy chain and surface expression of class I. This will be explored further in chapter 6. Lentivirus expressing β_2m-B44 has also been confirmed to be proficient in transduction and expression of β_2m-B44 protein and having confirmed this, β_2m-B44 virus and other viruses can be used in other cell lines such as tapasin or TAP deficient cell lines (see section 5.3.3).

4.3.3 Functional characterisation of various class I linked chain proteins in HCT cells

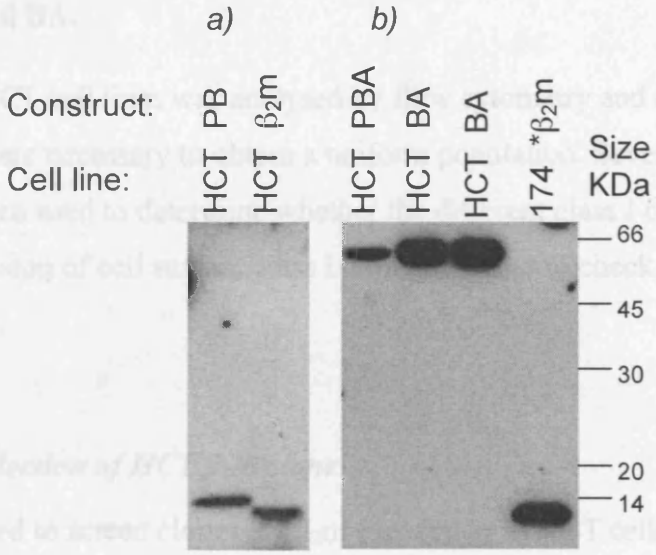
HCT cells are derived from a human colon carcinoma cell line, which is β_2m deficient and hence displays no surface MHC class I (Dexter et al., 1979). This HCT cell line was chosen for this study, because it is deficient in β_2m, but endogenously expresses heavy chain alleles including HLA-A2 (Bicknell et al., 1994). The β_2m deficiency is useful because it means that the introduced constructs can easily be detected using antibody against β_2m and also, because the HCT cell displays no cell surface MHC class I there is no interference from native class I in the results. The presence of HLA-A2 is necessary for the study of the PB construct whose peptide is derived from flu matrix peptide and is a natural binding epitope of HLA-A2.

HCT cell lines expressing PB, BA and PBA were provided by Dr. Bin Gao and to complement these an HCT line expressing β_2m was produced using the lentivirus pL-β_2m. HCT cells were transduced with lentivirus and stably selected for 10 days using blasticidin at 5μg/ml. 6 surviving colonies were successfully expanded for further analysis. (For details of constructs see Fig. 4.1).

4.3.3.1 Class I constructs were successfully expressed in HCT cells.

HCT, HCT PB, HCT BA and HCT PBA cells obtained from Dr. Bin Gao and HCT cells transduced with β_2m and selected with blasticidin were analysed by western blot for expression of the introduced protein using BBM.1 antibody (Fig. 4.13).

Figure 4.13. The four class I constructs can be detected in HCT cell lines. *.174 cells or HCT cells stably expressing the construct indicated were lysed, separated by SDS-PAGE and analysed by western blot with BBM.1 antibody. Except HCT BA, cells were loaded at 4 x 10^5 cells per lane. HCT BA cells were loaded with 1x10^5 cells. Membrane was probed with BBM.1 and α-mouse HRP. Film (a) was exposed for 10 minutes, film (b) was exposed for 1 minute. * Indicates native expression of protein.*



The western blots show successful expression of all four constructs in HCT cells. Lane 2 of Fig. 4.13a shows HCT cells in which normal β_2m has been restored. It can be seen from the other lanes, that wild type β_2m is absent in the other HCT cell lines, but is expressed strongly in the B cell line .174. The PB construct is 24 amino acids longer and can be seen as a band of slightly larger size than wild type β_2m. The BA construct consisting of both β_2m and the heavy chain allele HLA-A2 is seen as a band of significantly higher molecular weight (~50kDa), and the PBA construct is a similar size (Fig. 4.13b). The PBA construct is 24 amino acids longer than BA but because of the larger molecular weight of these molecules compared to β_2m and PB, this difference is not significant enough to see a clear difference in the size of the bands by separation using 12% SDS PAGE. The level of BA protein detected is significantly higher than the expression of the smaller proteins PB and β_2m in HCT cells, despite loading 4 times fewer cells and exposing the film for less time. This was repeatedly the case in western blots and shows that the expression level of BA is very high. All the constructs are under the control of the CMV promoter, so there is no reason to expect that the transcription rate for the BA protein is higher than the other constructs. It is more likely that the greater level of expression reflects high stability of the BA construct in HCT

cells. However, the copy number of the integrated plamids within each cell line is not known line and this cannot be ruled out as a possible influence on the protein expression level.

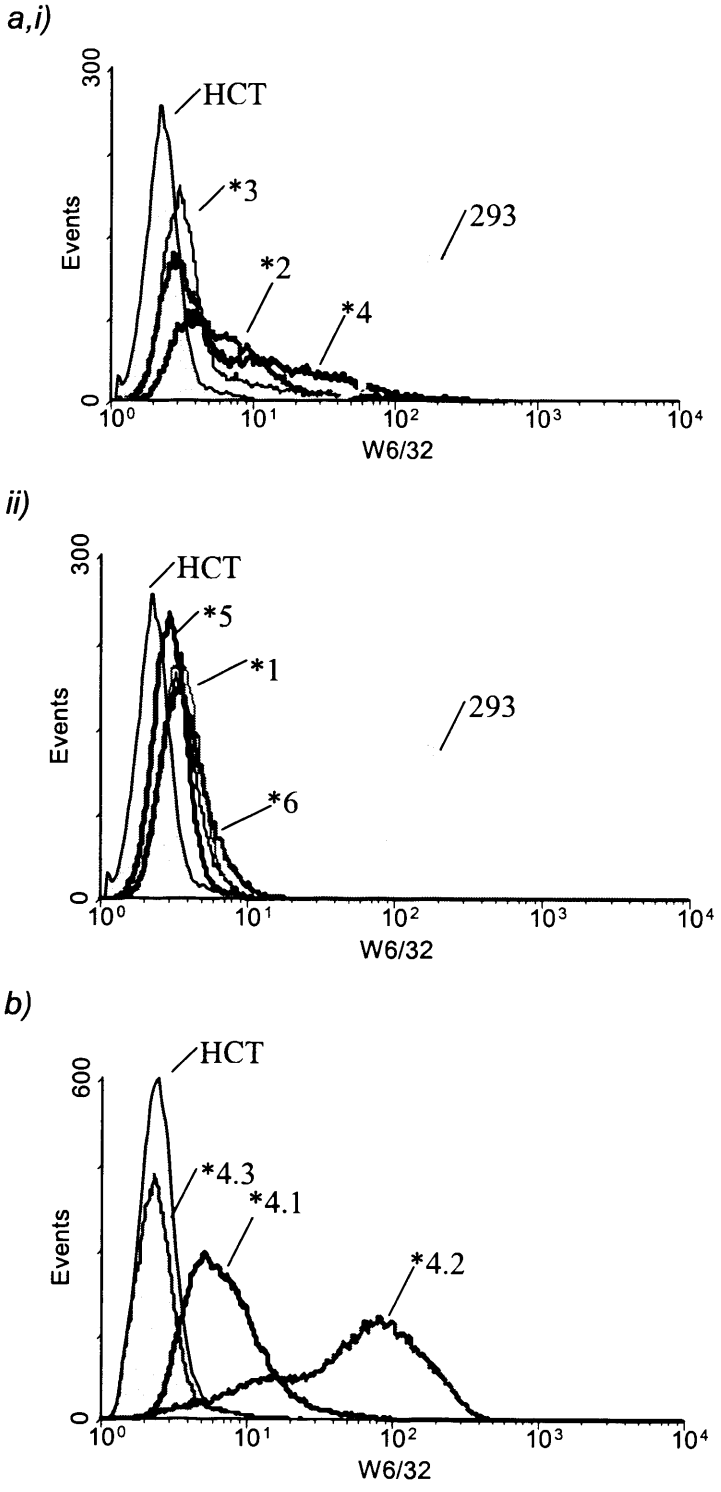
4.3.3.2 Cell surface MHC class I expression in HCT cells is restored by β_2m, PB and BA.

Each of the HCT cell lines was analysed by flow cytometry and drug selected or subcloned where necessary to obtain a uniform population. Several different antibody stains were then used to determine whether the different class I constructs were able to restore expression of cell surface class I expression and to check the folding status of the constructs.

4.3.3.2.1 Selection of HCT β_2m clone

FACS was used to screen clones for β_2m expression in HCT cells transduced with the lentivirus expressing human β_2m. After transduction of HCT cells with hβ_2m virus and expansion of individual blasticidin resistant colonies cells were stained with W6.32 antibody (recognising folded human class I molecules) and analysed by flow cytometry. Three "clones" (clones 2,3 and 4) showed clear cell surface expression of class I in 40%, 20% and 60% of cells respectively but appeared to have a mixed population of high and low expressers (Fig. 4.14a i). The other clones seemed to have only a slight increase in surface class I expression (Fig. 4.14a ii). Since good expression of β_2m in the HCT cell line should fully restore surface class I, the high expressers were thought to be those successfully transduced with β_2m virus. To obtain a pure population of high expressers, a cell sort was performed by flow cytometry. Clone 4 was sorted on a gate to include only the top 20% cells expressing class I. Cells were plated in a 96 well plate at an average of 0.5 cells per well to carry out limited dilution cloning. New subclones were grown up from single cells and screened for their surface expression of class I. From the single cell sort of clone 4 the high expressing clone '4.2' was chosen for use in future experiments as it expressed high levels of cell surface class I (Fig. 4.14b).

Figure 4.14. A population within HCT β_2m clones 2,3 and 4 expressed surface class I, and subclone 4.2 displays good expression of class I. *HCT cells (shaded), HEK 293 cells (light grey) and HCT β_2m clones were stained with W6.32 antibody and α-mouse PE and analysed by flow cytometry. a) i) HCT β_2m clone 2 (dark grey -*2), clone 3 (thin black -*3) and clone 4 (thick black -*4) ii) HCT β_2m clones 1,5 and 6 (*1, *5 and *6). b) Further subclones of clone 4. Clone 4.1 (thick black - *4.1), 4.2 (dark grey - *4.2), 4.3 (black - *4.3)*



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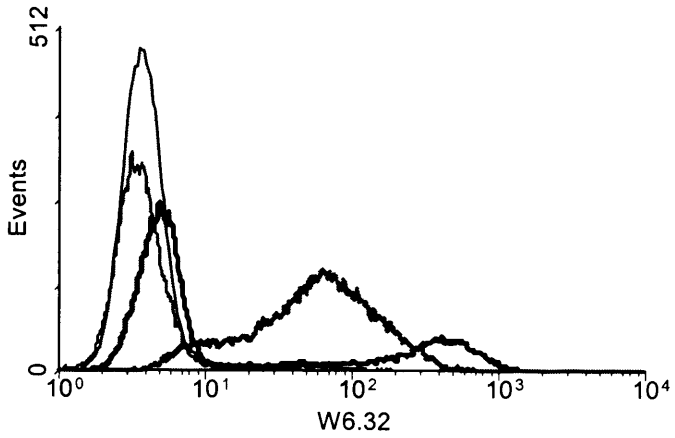
4.3.3.2.2 PB cells were selected to obtain a pure population

The HCT PB cell line obtained from Dr. Bin Gao was thawed and allowed to recover. The PB construct had been introduced into the HCT cell line using a plasmid encoding a zeocin resistance gene. Cells were assessed for expression of the PB construct by staining with W6.32 and analysing by flow cytometry. However, expression of class I in the HCT PB cell line was found to be unstable when grown in zeocin free medium. About 20% of cells expressed β_2m at the cell surface when thawed from stock the HCT PB cell line. However, this expression was rapidly lost within 10 days if grown in zeocin free medium. When grown in medium containing 50μg/ml zeocin, initially most of the cells died and growth of surviving cells was slow. However, after maintenance in zeocin for 6 weeks, cells were dividing at a rate typical for HCT cells (about 1 division every 24 hours) and cell death was low (less than 5%). When assessed by flow cytometry using W6.32 antibody a high level of expression was detected at the cell surface of all zeocin treated cells indicating that the cells expressing PB had been positively selected (Fig. 4.15).

Figure 4.15. A uniform population of HCT PB cells expressing surface class I was obtained by treating cells with zeocin.
HCT PB stock cells were thawed and grown in: zeocin free medium and assessed 24 hours later (thick black), zeocin free medium for 10 days (thin black) or grown for six weeks in medium containing zeocin at 50μg/ml (thick grey). HCT cells – shaded. Cells were stained with W6.32 and α-mouse PE and analysed by flow cytometry.

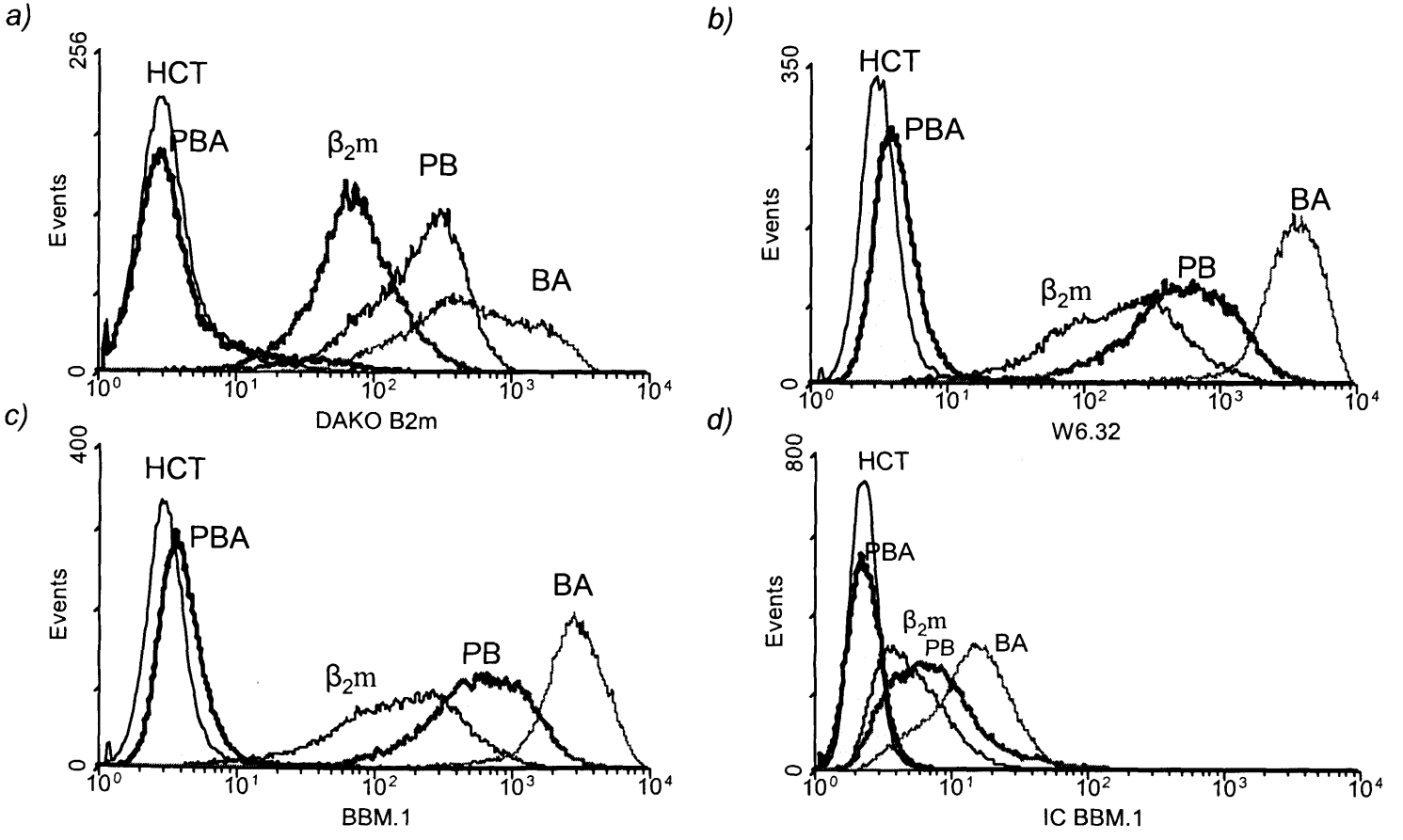


4.3.3.2.3 Stable expression at the surface of HCT β_2m, HCT PB and HCT BA is confirmed by staining with a panel of class I specific antibodies.

The expression of human MHC class I on the surface of the 5 HCT cell lines was assessed by flow cytometry using purified polyclonal α-hβ_2m (DAKO), the monoclonal α-hβ_2m antibody BBM.1, the monoclonal antibody W6.32, which recognises folded β_2m-HC heterodimer (Fig. 4.16 a-c) and the monoclonal antibody BB7.2, which recognises HLA-A2 (data not shown). The antibodies all show a similar staining pattern, with β_2m, PB and BA all restoring expression of MHC class I, but PBA showing little or no expression. The linked chain constructs PB and BA both express at high levels, and consistent with western blot results BA was consistently found at a level at least 10-fold higher than HCT cells expressing β_2m.

The staining of HCT PBA cells is weak with all antibodies. A marginal shift in cell surface expression was typically observed with all antibodies but it is hard to be sure that such a small shift reflects genuine cell surface expression of the PBA construct. Intracellular staining with BBM.1 and flow cytometry also fails to detect a significant shift in PBA expression (Fig. 4.16d) despite the fact that it is detectable by western blot using BBM.1 (Fig. 4.13b). It is surprising that PBA cannot be recognised by intracellular staining given that BBM.1 recognises a linear epitope within β_2m and the other constructs can all be stained with BBM.1. However, it may be that the true intracellular staining with BBM.1 is weak and that the shift observed for intracellular staining of HCT BA and other cell lines is a reflection only of the surface class I and is not actually intracellular staining.

Figure 4.16. A panel of antibodies confirms expression and folding of class I constructs on the surface of HCT cell lines. *a-c) HCT cells and HCT cells expressing β_2m, PB, BA or PBA were stained with polyclonal α-hβ_2m (DAKO) (a), conformation dependent α-class I mAb W6.32 (b), or α-hβ_2m mAb antibody BBM.1 (c) and analysed by flow cytometry. d) Cells as in (a) were permeabalised and stained with BBM.1 antibody and analysed by flow cytometry. HCT* - HCT, *HCT PBA* - PBA, *HCT β_2m* - β_2m, *HCT PB* - PB, *HCT BA* - BA.



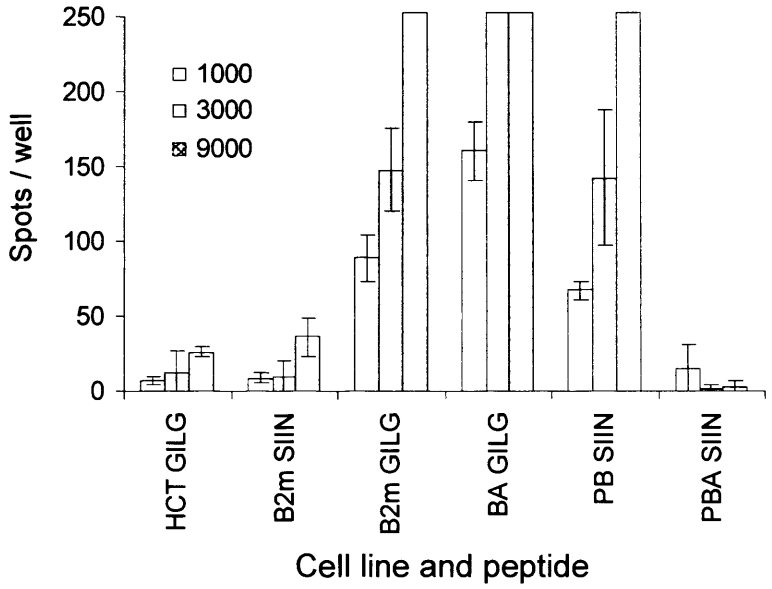
4.3.3.3 Class I fusion proteins PB and BA are functional in terms of antigen presentation to T cells.

Expression of either PB or BA in HCT cells recovered the defect in cell surface expression of class I in HCT cells, showing that both PB and BA constructs are able to express at the cell surface. However, the function of class I molecules is to present antigens to T cells, and although recovery of class I expression is important, it does not prove that these constructs are able to present antigen. Using a CTL clone specific for flu matrix peptide GILGFTFVL in association with HLA-A2 (CTL_{FLU}) (kindly provided by Dr. Xiaoning Xu, Institute of Molecular Medicine, Oxford), it is possible to assess how effectively each of the MHC class I constructs presents antigen to T cells. The PB and PBA constructs include the flu matrix peptide in their sequence and recognition by CTL_{FLU} would show that these constructs are able to fold normally and that the peptide sits correctly in the HLA-A2 peptide binding groove. The other constructs can be assessed for their ability to present antigen by pulsing the cells with flu peptide prior to incubation with the CTL_{FLU}.

Upon activation, T cells produce cytokines such as IFNγ and by using the ELISpot assay to measure IFNγ production, it is possible to determine the number of cells stimulated by the antigen presenting cells (see section 2.5.2). To determine whether class I constructs were able to present antigen to T cells, HCT cells expressing fusion proteins were tested for their ability to stimulate IFNγ production by in CTL_{FLU}. Antigen presenting cells (HCT cell lines) were pulsed with either flu matrix peptide or the irrelevant ovalbumin derived peptide SIINFEKL, at 100μM for 1 hour at 37°C, washed 3 times to remove free peptide and then 50,000 cells were used to stimulate 0, 1000, 3000 or 9000 CTL_{FLU} cells.
As expected, untransfected HCT cells were unable to stimulate additional IFNγ production by CTL_{FLU} even when pulsed with flu peptide (Fig. 4.17). HCT PB cells stimulated strongly regardless of the peptide provided indicating that the peptide attached to the PB construct sits in the peptide groove and can present effectively to T cells. HCT β_2m and BA cells were both able to stimulate CTL_{FLU} when pulsed with flu peptide GILGFVFTL but not when pulsed with irrelevant peptide. The IFNγ response of CTL_{FLU} to HCT β_2m pulsed with GILGFVFTL and HCT PB cells is similar indicating that cells expressing PB are able to present specific antigen as efficiently as cells expressing normal class I molecules. The antigen specific response of CTL_{FLU} to

HCT BA cells is enhanced nearly 2-fold over HCT β_2m cells indicating that BA may be able to present antigen more efficiently than wild type class I. This may be influenced by the higher level of cell surface expression of class I in HCT BA cells.

Figure 4.17. PB can stimulate T cells as effectively as normal class I and BA can stimulate a stronger response.
HCT cells (HCT) and HCT cells expressing β_2m (B2m), or the class I fusion proteins PB (PB), BA (BA) or PBA (PBA) were pulsed with either 100μM flu matrix peptide GILGFVFTL (GILG) or 100μM irrelevant peptide SIINFEKL (SIIN) for 1 hour at 37°C. 5 x 104 targets were then incubated with CTL_{FLU} overnight. IFNγ producing cells were measured by ELISpot. Data shown is the mean and standard deviation of 4 wells in one of 3 similar experiments. (Limitations of the experiment mean that a maximum of 250 spots may be counted per well.)

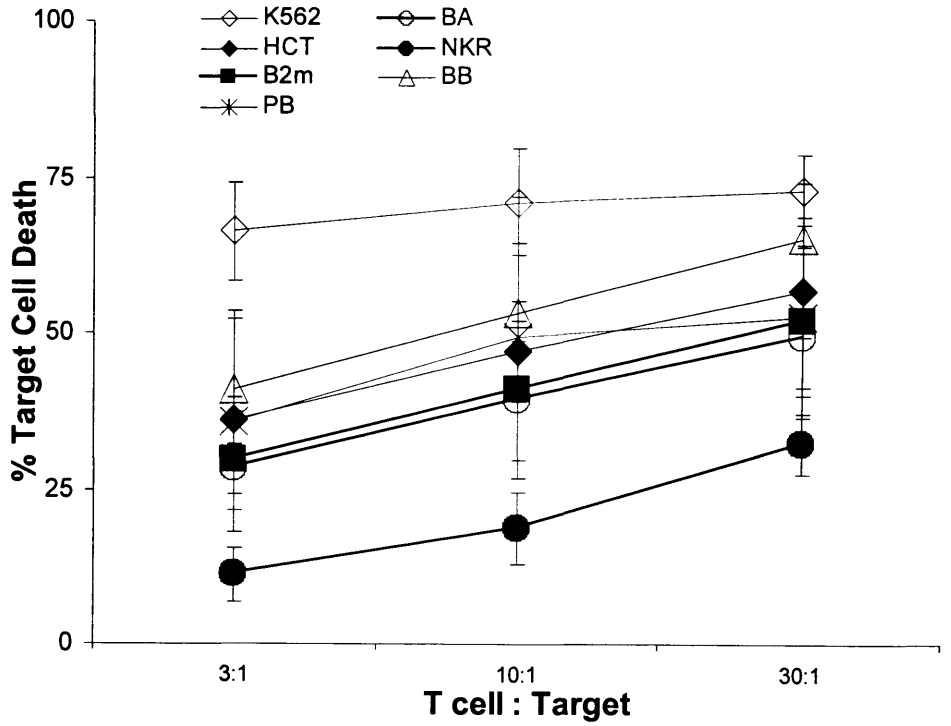


4.3.3.4 NK92 Cell killing is not significantly changed by the expression of class I constructs on HCT cells.

The NK like cell line, NK92 was used primarily to optimise the Cr^{51} cell killing assay, since unlike specific T cells, which can be difficult to maintain, NK92 cells are easy to grow. Additionally, it was interesting to test whether HCT cells were particularly susceptible to NK92 killing because of their deficiency in β_2m and whether restoring class I expression either with normal β_2m or with linked chain constructs affected the rate of killing. NK92 susceptible K562 cells were used as a positive control and the resistant CEM.NKR cells (NKR) as a negative control. Target cells were assessed for susceptibility to killing by analysing the induced chromium release after a 4 hour

incubation with NK92 cells (Fig. 4.18). As expected K562 cells were killed effectively by NK92 cells, with chromium release of over 65% even at a NK92 to target cell ratio of 3:1. At this NK92 to target cell ratio, killing of NKR cells is only 12% and rises only to 30% at a ratio of 30:1. Killing of HCT cells by NK92 was significantly lower than killing of K562 cells but significantly higher than killing of NKR cells. Killing of HCT cells expressing BA or PB constructs (where cell surface class I expression is restored) was slightly reduced in two of three experiments performed, but the difference was not found to be significant. Killing of HCT β_2m and HCT β_2m-B44 cells was very similar to HCT cells. Overall the results indicate that although HCT cells are susceptible to NK92 cell killing, surface class I expression in HCT target cells does not have a significant influence on their susceptibility to NK92 killing.

Figure 4.18. Expression of β_2m or class I fusion proteins in HCT cells has no effect on susceptibility to NK92 killing.
K562, NKR, HCT cells and HCT cells expressing β_2m (B2m) or the class I fusion proteins PB, BA or BB were labelled for 1 hour with Cr^{51}, washed thoroughly and incubated for 4 hours with NK92 cells at the ratio indicated. Target cell death was estimated from the % Chromium release, normalised to take into account spontaneous release. Triplicate data are pooled from 3 independent experiments and are shown as mean and 95% confidence intervals.



4.3.3.5 BA and PB constructs can be specifically recognised and killed by CTL_{FLU}.

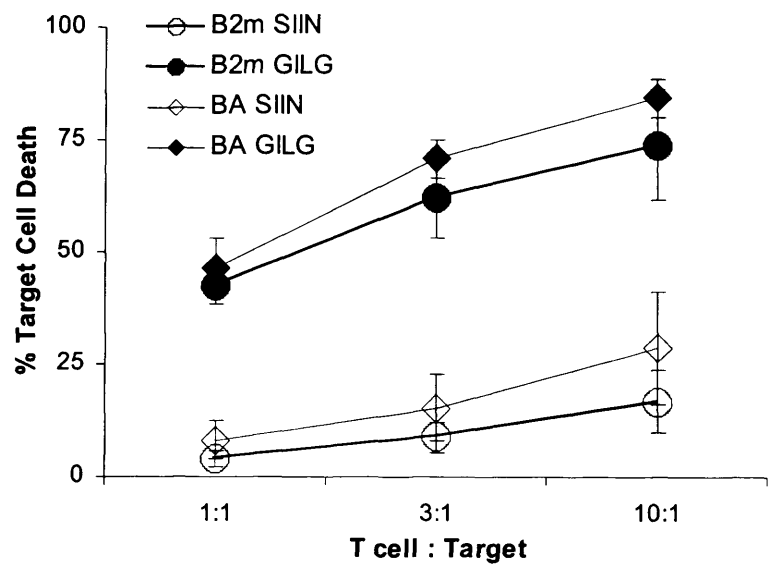
Once the conditions of chromium loading and incubation had been established in killing assays with NK92 cells, CTL_{FLU} were assessed for their ability to kill HCT cells expressing the different class I constructs. As expected when the β_2m-/- HCT cell line was pulsed with flu peptide it was not killed by CTL_{FLU} as measured by Chromium release (Fig. 4.19a). However, expression of either β_2m or BA not only restored cell surface expression of class I but also allowed cells to present specific peptide to CTL_{FLU}. In the chromium release assay when these cells were pulsed with flu peptide up to 80% of them were killed by CTL_{FLU}, whereas irrelevant peptide induced no increase in killing (Fig. 4.19a). This confirms that not only is the BA construct able to fold and reach the surface, but also that peptide is able to bind to the binding groove in the usual conformation for recognition by T cells.

The PB construct shows similarly positive results, here there is no need to pulse the cells with peptide because the construct contains both β_2m and flu matrix peptide, in the experiment shown in figure 4.19b HCT PB cells were pulsed with an irrelevant peptide, but CTL_{FLU} could be stimulated equally efficiently without addition of any peptide (Fig. 5.4). In the chromium release assay CTL_{FLU} is able to kill HCT PB cells as efficiently as HCT β_2m cells pulsed with flu peptide (Fig. 4.19b). These results show that the PB construct is functional, in other words that it can pair up correctly with HLA-A2 and fold into a mature class I molecule with the peptide lying correctly in the binding groove of the heavy chain.

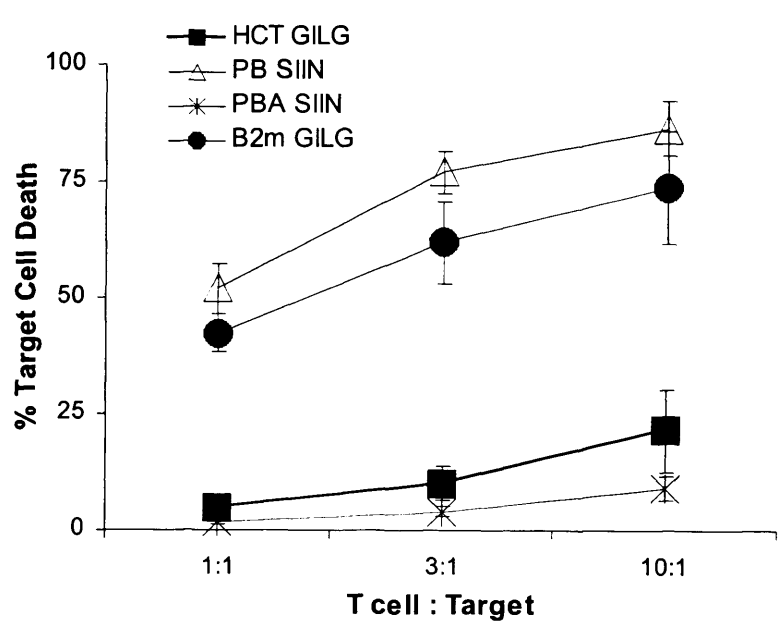
In contrast cells expressing PBA are unable to elicit any response from CTL_{FLU} presumably reflecting the failure of this construct to reach the cell surface (Fig. 4.19b).

Figure 4.19. HCT cells expressing class I fusion proteins can be specifically killed by CTL_{FLU}.
Cells were labelled with Cr^{51} and simultaneously pulsed with peptide. Cells were then incubated with CTL_{FLU} for 4 hours at the indicated T- cell to Target ratios. Target cell death was estimated from the % Chromium release normalised to take into account spontaneous release. Data shown are pooled from at least 3 independent experiments and show mean and 95% confidence intervals. a) HCT cells expressing β_2m (B2m) or BA (BA) pulsed with flu peptide GILGFVFTL (GILG) or the irrelevant peptide SIINFEKL (SIIN) as indicated. b) HCT cells (HCT) or HCT cells expressing β_2m (B2m), PB (PB) or PBA (PBA) pulsed with with flu peptide GILGFVFTL (GILG) or the irrelevant peptide SIINFEKL (SIIN) as indicated.

a)



b)

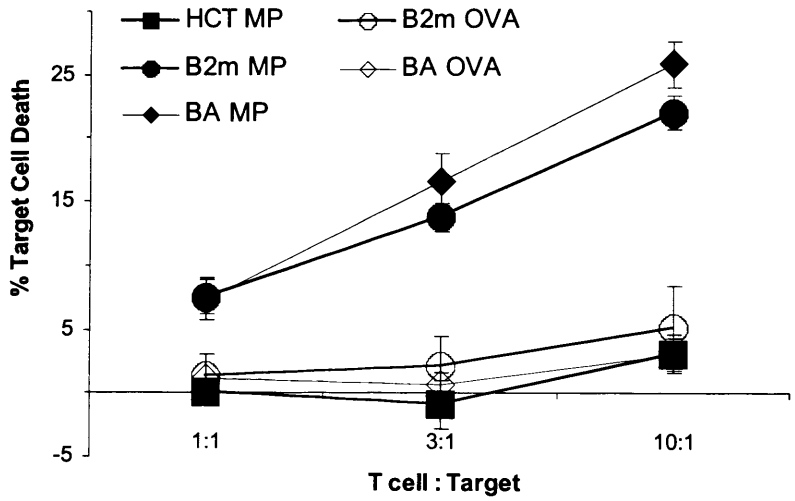


4.3.3.6 Endogenously processed antigen can be presented as efficiently by the linked chain class I molecule 'BA' as native class I.

To allow an effective immune response to occur *in vivo*, cells must be able to process antigen from whole protein and then load the antigenic peptide on to the developing class I molecule in the ER. In order to check that the BA construct can present such endogenously processed antigen, cells were infected with vaccinia virus expressing full length flu matrix protein (V-MP). In order to be recognised by CTL_{FLU}, cells must now be able process whole protein into peptide antigens and load them on to developing class I molecules in the ER.

When infected with V-MP, HCT β_2m cells but not HCT cells were able stimulate specific T cells resulting in recognition and killing (Fig. 4.20). HCT BA cells were also able to stimulate killing showing that they were able to process antigen correctly and present peptide derived from endogenously expressed flu matrix protein to specific T cells (Fig. 4.20). The presentation of endogenous antigen to T cells induces a less powerful response than cells pulsed with peptide. However, there is still a significant difference in the rate of killing of either HCT β_2m or HCT BA cells infected with vaccinia virus expressing flu matrix protein compared to those infected with vaccinia virus expressing the irrelevant protein ovalbumin. The ability of BA to present endogenously processed antigen shows that peptide loading of BA can occur efficiently in the ER as well as at the cell surface.

Figure 4.20. The BA construct can present endogenously processed antigen and induce T cell killing.
HCT cells (HCT) and HCT cells expressing β_2m (B2m) or the class I fusion protein BA (BA) were infected with vaccinia virus expressing either Flu matrix protein (MP) or the irrelevant protein ovalbumin (OVA). After infection cells were labelled with Cr^{51} and incubated with CTL_{FLU} for 4 hours at the indicated T- cell to Target ratios. Target cell death was estimated from the % Chromium release normalised to take into account spontaneous release. Data shown are pooled from at least 3 independent experiments and show mean and 95% confidence intervals.



4.4 Discussion

In the work shown in this chapter, a viral system for delivery of class I fusion proteins has been developed and validated, and stable cell lines expressing fusion proteins have been created in both human and mouse cell systems. Furthermore, the class I fusion proteins PB and BA have been shown to express at the cell surface in human cells and to present antigen to an influenza specific CTL clone.

A 5 cell HCT system – HCT, HCT β2m, HCT PB, HCT BA and HCT PBA has now been created and optimised. The three fusion proteins upon synthesis, are at different stages of folding into a fully assembled class I molecule containing peptide, heavy chain and β_2m, and represent an interesting prospect for research into class I folding and assembly. In this chapter β_2m, PB, BA and PBA have been successfully expressed in HCT cells. Of these, β_2m, PB and BA are able to express at the cell surface but PBA is not.

The PB construct restores expression of class I molecules at the surface of β_2m deficient HCT cells. In order to do this it must pair up in the normal way with HLA-A2 heavy chain (to which the peptide binds) and traffic to the cell surface. The W6.32 antibody recognises folded class I β_2m-HC heterodimer and positive staining of HCT PB cells with W6.32 confirms that the PB construct is able to form a native structure when paired with endogenous heavy chain. Comparison of BBM.1 and W6.32 staining indicates that the proportion of correctly to incorrectly folded β_2m-HC heterodimer at the cell surface is similar in HCT PB cells to that in HCT β_2m. The level of expression is found to be higher for HCT PB than HCT β_2m and together these findings imply that expression and folding of PB with heavy chain is stable. In order to present its peptide correctly to T cells, PB must not only fold with HLA-A2, but also the peptide must sit correctly in the peptide binding groove. T cell stimulation and T cell killing assays both show that the PB construct is able to effectively present to the T cell clone CTL_{FLU} without provision of peptide, proving that the peptide of the PB construct does indeed sit in the peptide groove of HLA-A2.

There are several possible applications for PB both in dissecting the mechanisms of class I antigen processing and in T cell stimulation. To date there has been a large amount of interest in the mechanisms of peptide binding and optimisation and of the

chaperones involved (Howarth et al., 2004;Zarling et al., 2003;Paquet et al., 2004). The quality control mechanism of class I loading is thought to allow trafficking of properly loaded class I molecules, whilst retaining those that are empty or loaded with low affinity peptide. There is some evidence that this may be achieved by peptide triggered release of class I molecules from the peptide loading complex (Knittler et al., 1999;Suh et al., 1994). The PB construct provides a valuable tool for analysing this theory and this is explored further in chapter 5.

Since it has been shown to stimulate specific CTL, PB or similar constructs could be used to expand T cells *in vitro*. This could have a distinct advantage over normal peptide stimulation, since the stimulation could be more powerful and longer lasting, although in this work stimulation was comparable in strength to β_2m expressing HCT cells pulsed with specific peptide. However, the stimulation of T cells with peptide pulsed feeder cells relies on the class I molecules of the feeder cells swapping the endogenously processed peptide for the exogenously administered peptide at the cell surface. Although this does seem to occur, the proportion of class I molecules expressing the desired peptide may be low and may be dependent on the peptide used. Furthermore, as class I molecules are turned over at the cell surface, the expression of molecules carrying the desired peptide will fall. In contrast, expression of PB in the antigen presenting cells should persist, resulting in steady cell surface expression of class I molecules carrying the desired peptide. In the case of HCT cells it is probable that all class I molecules will display the incorporated peptide since there is no competition by wild type β_2m. However, HCT may not be the cell line of choice for such work since it displays relatively low levels of class I compared with B cells or other professional antigen presenting cells.
Since, it has been shown in this chapter that expression of PB is stable at the cell surface, it could therefore provide more prolonged stimulation of T cells than peptide pulsed cells. The obvious disadvantage of such a system is that the peptide incorporated in PB must be changed for each peptide of interest and of course the correct class I allele must be carried by the antigen presenting cell. Nevertheless, PB constructs could be useful for the expansion of known peptide specific CTL or to detect peptide specific CTL within a PBMC or other mixed cell population.

Taking this idea one step further PB like constructs could be used for vaccination. The mechanisms of DNA vaccination remain poorly understood, but presumably involve

protein synthesis within cells. After DNA vaccination with PB, antigen presenting cells would be able to fold PB with the appropriate endogenously expressed heavy chain allele and present the peptide at the cell surface. Unlike presentation of traditional antigens this would not require any processing within the cell. This may increase the antigen presentation efficiency and boost T cell stimulation leading to a powerful immune response. Limited work has been carried out on such a vaccine strategy but this deserves further study (Uger et al., 1999).

FACS staining with W6.32 shows that the BA construct (β_2m linked to HLA-A2) is also able to fold correctly and traffic to the cell surface. It is highly expressed at the cell surface in HCT cells as shown by staining with a panel of class I antibodies. This 10-fold increase in surface expression may reflect a slower cell surface turnover rate of BA compared with normal MHC class I molecules. Previous work has shown that loss of peptide from class I destabilises the interaction between β_2m and heavy chain and results in internalisation of MHC class I (Ljunggren et al., 1990). Since heavy chain and β_2m are covalently linked in the BA construct, it is possible that it retains peptide more effectively or that class I remains more stable at the cell surface even after loss of peptide. This would lead to a lower turnover rate, a higher level of cell surface expression (as shown by flow cytometry) and a higher level of total expression (as shown by western blot).

The BA construct is able to fold correctly and become loaded with peptide, which it can present to T cells as efficiently or more efficiently that native MHC class I in β_2m expressing HCT cells. The slightly increased level of CTL_{FLU} stimulation and killing induced by BA may reflect the higher level of class I at the cell surface. Like normal class I molecules BA is able to present endogenously processed antigen as well as exogenously provided peptide. Although the rate of killing is fairly low (30%) in HCT BA cells when they are provided with whole flu matrix protein it is significantly higher than killing of cells expressing irrelevant ovalbumin protein. HCT BA cells appear to be slightly more susceptible to specifife T cell killing than HCT β_2m cells although the difference is not statistically significant. However, it demonstrates that antigen can be loaded onto the BA fusion protein in the ER at least as efficiently as native class I. The low level of killing in these experiments may reflect either incomplete infection of cells by vaccinia virus expressing flu matrix protein, or the fact that HCT cells express relatively low levels of chaperones and MHC class I and are therefore relatively poor

targets for T cell killing. B cell lines or PBMCs which are often used as targets in this type of work express much higher levels of these molecules and would therefore be more susceptible to T cell killing.

Linked chain constructs of β_2m and heavy chain may also provide useful tools in the future for detection of specific T cell receptors. Current tetramer technology requires production and *in vitro* refolding of β_2m heavy chain and peptide. This is a laborious process and conditions must often be optimised for each peptide or heavy chain allele required. The covalent linkage of β_2m and heavy chain could simplify refolding allowing tetramers to be synthesised more cheaply and easily. Single chain trimers such as PBA would potentially also be useful for this kind of work. Although the desired peptide would have to be cloned into the construct for each tetramer required the whole molecule could then be produced in bacterial or yeast systems. If the protein correctly folded in these systems, it would greatly reduce the cost and labour involved in the production of tetramer as no *in vitro* refolding would be required.

The fact that both the BA and the PB constructs are functional makes it surprising that the PBA construct (containing all three components of the folded class I molecule) is unable to traffic to the cell surface and present antigen. Intracellular staining with BBM.1 antibody also fails to detect the PBA complex although it is shown to be present by western blot. One possible explanation for this is that it folds incorrectly in the ER causing chaperone molecules to bind it and hiding the epitope normally recognised by BBM.1 antibody. Detection by western blotting is still possible because as the proteins are denatured, chaperone interactions are disrupted and the epitope becomes available. It is not clear why PBA fails to traffic to the cell surface, but it is possible that there is some steric problem in folding the molecule in its current form. It may be that expressing the fragments in a different order or with different linkers would allow expression of a complete single chain MHC class I molecule (Yu et al., 2002).

By introducing β_2m-B44 (BB) into the murine cell line K41 and its calreticulin deficient counterpart K42 using lentivirus, a model has been created to assess the function of calreticulin and other species specific components in the trafficking of class I molecules. It is widely accepted, that when class I heavy chain is newly synthesized, it associates with calnexin, which maintains it in a β_2m receptive state (David et al., 1993). When β_2m is recruited to associate with class I heavy chain, this is accompanied

by the replacement of calnexin with calreticulin (Sadasivan et al., 1996). Although the mechanism of the association of β_2m and heavy chain and the simultaneous replacement of calnexin with calreticulin is not known, given the sequence of protein interactions it is possible that calreticulin may be responsible either for promoting the assembly of class I heavy chain with β_2m or for the recruitment of β_2m. If calreticulin plays an important role in the formation of the β_2m-HC heterodimer, then this might explain the reduced cell surface expression of class I in the K42 (crt -/-) cell line. By linking β_2m and heavy chain together the requirement for β_2m recruitment would be lost and class I expression might be restored in K42 cells. Similarly the BA construct might be useful in the study of the recruitment of β_2m to heavy chain. A comparison of BB (β_2m linked to HLA-B44) and BA might also reveal allele specific differences in regards to β_2m recruitment; these ideas are explored further in chapter 6.

HLA-B44 virus was also created in this chapter, primarily for use as a control for the linked chain molecule β_2m-B44 (BB) as this was expected to be useful in an assessment of the importance of chaperone molecules in the recruitment of β_2m to heavy chain. Because alleles of class I are known to vary in respect of peptide loading especially in their dependency on tapasin, the choice of class I allele is important. HLA-B*4402 is known to be particularly dependent on tapasin for its expression (Peh et al., 1998). This has been attributed to the presence of an aspartic acid at residue 116 located in the peptide groove; HLA-B*4405 in which this residue is replaced with tyrosine, is known to traffic more independently of tapasin (Williams et al., 2002). For the purpose of this study it is useful to study HLA-B*4402, since its high dependency on tapasin and the PLC may mean that any enhancement of expression by linking β_2m to heavy chain is more noticeable for HLA-B44 than for other alleles.

The work in this chapter provides the basis for further experimentation. By showing that the fusion proteins PB and BA express and fold correctly and that they are fully functional it is reasonable to use such constructs to investigate their interactions with molecular chaperones. The generation of both murine and human cells stably expressing fusion proteins will facilitate this study and the versatility of the lentivirus system allows transduction of other cells when necessary.

5 Fusion proteins indicate a default pathway for class I maturation unaffected by peptide binding and β_2m recruitment.

5.1 Introduction

In human cells, the generally the accepted model of MHC class I trafficking is that heavy chain (HC) binds to calnexin during and shortly after synthesis in what is commonly known as the 'early complex' (Degen and Williams, 1991;David et al., 1993). Following binding of β_2m to heavy chain, calnexin is dropped in favour of calreticulin (Sadasivan et al., 1996). After the β_2m-HC class I heterodimer has formed, it associates with the peptide-loading complex, which facilitates binding of high affinity peptide (Ortmann et al., 1994;Sadasivan et al., 1996;Zarling et al., 2003). Once high affinity peptide is bound to class I, it is released from the peptide-loading complex (Suh et al., 1994). Because release of class I is conditional on the binding of high affinity peptide this provides a mechanism for quality control of class I peptide loading.

In this chapter, partially preassembled class I molecules 'peptide linked β_2m' (PB) and 'β_2m linked heavy chain' molecules β_2m-A2 (BA) and β_2m-B44 (BB) (see Fig. 1.4) are used to investigate this pathway. The theory that peptide binding triggers the release of class I from the peptide-loading complex is investigated, by studying the interaction of PB with tapasin and TAP. The trafficking rate of PB and BA constructs is examined by pulse chase to determine whether the fact that they are partially preassembled allows them to fold and leave the ER faster than normal class I molecules. The dependence of class I β_2m-HC heterodimers on the peptide-loading complex is then investigated, by examining their interaction with tapasin and TAP and by expression in tapasin and TAP deficient cell lines. Finally, the release of class I from the 'early complex' is examined by investigating the interaction of calnexin with normal heavy chain and with the linked chain construct BA.

Numerous approaches have previously been used to investigate the processes involved class I folding, from the synthesis of MHC class I in the ER to the presentation of antigenic peptide at the cell surface. These approaches fall broadly into three categories, which may be used on their own or in concert. Firstly, the study of associations between proteins involved in class I loading and antigen processing, typically using the technique of immunoprecipitation. Secondly, the study of cells, which display an abnormal phenotype with respect to MHC class I antigen processing, because of a

deficiency in one of the proteins involved. Thirdly, modification of heavy chain, or other components of class I loading, resulting in a change in its interactions with other proteins and an altered phenotype (e.g. HLA-A2 mutant T134K (Lewis et al., 1996)). Here a novel approach is presented: MHC class I fusion proteins that are partially assembled upon synthesis are examined in terms of their trafficking and peptide loading. By linking together the three different pieces of a mature MHC class I trimeric complex consisting of HLA-A2, β_2m and the flu matrix protein derived peptide GILGFVFTL (Fig. 1.4), it is possible to investigate the interactions between MHC class I and different chaperone molecules along the process of maturation.

The human HCT cell line, which is deficient in β_2m and therefore displays no cell surface MHC class I, is a good model cell line to use in this work. The β_2m deficiency is convenient as it allows easy antibody detection and isolation of the introduced constructs without interference from native β_2m. Additionally, in the absence of β_2m, heavy chain does not associate with the peptide-loading complex, but is instead degraded before leaving the ER (Williams et al., 1989). Therefore, it can be deduced that any class I entering the peptide-loading complex is doing so as a consequence of the introduced class I construct. The genotype of the HCT cell line includes HLA-A2 (Bicknell et al., 1994), which is required for the study of the PB construct whose peptide is derived from flu matrix protein and is a natural binding epitope of HLA-A2.

The technique of immunoprecipitation (IP) can be used to isolate proteins and under the correct conditions to study interactions between proteins. In this work immunoprecipitation is used to analyse interactions between different molecules involved in MHC class I antigen processing. However, most common detergents disrupt the interactions between class I and members of the peptide-loading complex, so care must be taken with the conditions of lysis to maintain protein associations. One of the most significant investigations of the interactions between class I and its associated chaperones was carried out by Sadasivan et al (Sadasivan et al., 1996), in which they showed that not only is β_2m-HC heterodimer bound by calreticulin, but also identified "a novel uncharacterized 48 kDa glycoprotein, tapasin, which can bind independently to both TAP and class I-β_2m-calreticulin complexes.". In this and other studies 1% digitonin is used as lysis buffer and so a similar protocol has been followed in the work presented here.

Cell lysates are then incubated with antibody or antiserum against the target protein; the choice of antibody is important since the interaction must be of high affinity and the epitope must be available to the antibody when the protein is itself complexed with other proteins. In this work polyclonal rabbit serum has generally been used, which can be more reliable since it is not dependent on any one particular epitope of the protein in interest. Antibody – protein complexes are then purified using protein G or protein A beads, which bind to the antibody. Protein A and protein G are structural polypeptides of bacterial cell walls. Protein A was identified first and was isolated from *Staphylococcus aureus* and was shown to bind to antibody molecules (Forsgren and Sjoquist, 1966), hence it can be used to purify antibody from solution. Protein G was subsequently purified from group G *Streptococci* and protein A and protein G were shown to have distinct but overlapping binding properties for the Fc portion of antibodies(Langone et al., 1978;Kronvall et al., 1970).
Once the target protein has been 'immunoprecipitated' the complex is washed extensively and the proteins of interest are eluted, usually by low pH or SDS. Detection of eluted proteins may be done specifically by immunoblotting (using an antibody or antiserum of a different species from the IP antibody), or cells can be labelled with radioactive amino acids prior to lysis and detected using photographic film, following SDS PAGE or two-dimensional electrophoresis (O'Farrell et al., 1977).
In this work, immunoprecipitation is used to identify the interactions occurring between class I fusion proteins and other proteins of the class I processing pathway. These interactions can then be compared with those made in cells expressing wild type class I molecules.

In order to examine the rate of class I export from the ER, the technique of pulse chase has been used. In this technique, proteins are radioactively labelled during synthesis and then immunoprecipitated at various time intervals to follow their trafficking. Cells are starved of methionine (met) and cysteine (cys) for 60 minutes and then incubated for 15 minutes with met and cys labelled with S^{35} for 15 minutes. During this time proteins synthesised incorporate the S^{35} label allowing them to be 'chased' for a period of time afterwards. The target protein, in this case heavy chain, is isolated from lysates at various time points after labelling using immunoprecipitation with specific antibody and protein G purification. By treating the isolated heavy chain with endoglycosidase H (endo H), it is possible to detect whether it has exited the ER and passed through the trans-Golgi network.

This technique relies on the fact that proteins such as class I heavy chain, which are N-glycosylated in the ER, are susceptible to sugar cleavage by endo H enzyme. As a result when treated with endo H the molecular weight is decreased and the protein has higher mobility on SDS separation than untreated or endo H resistant proteins. However, in the Golgi network, the sensitive high mannose sugar residues are modified to complex forms and susceptibility to endo H is lost. By analysing the proportion of endo H sensitive to endo H resistant labelled protein at different time points, it is possible to estimate the average length of time that the protein spends in the ER before trafficking through the Golgi body.

In this chapter, class I fusion proteins will be used to examine the processes of class I loading. The process of class I loading may be thought of like an assembly line. After synthesis heavy chain heavy chain interacts with calnexin in the 'early complex', once it has assembled into the β_2m-HC heterodimer it progresses to the peptide-loading complex. After high affinity peptide is bound, the trimeric class I molecule is complete and can be released from the peptide-loading complex and can traffic to the cell surface. Since class I fusion proteins are partially assembled their full assembly may no longer require every stage of the 'assembly line'. The BA construct for instance may no longer interact with calnexin since the heavy chain allele HLA-A2 is covalently linked to β_2m in this construct. On the other hand since no peptide is present it would still be expected to travel via the peptide-loading complex. In contrast the PB construct in which peptide is covalently linked to β_2m would be expected to have no requirement for the peptide-loading complex. Once PB associates with HLA-A2 it already contains all three components of the complete class I molecule and so it may be able to traffic directly to the cell surface.
These ideas are tested in this chapter using pulse chase, immunoprecipitation, and by expression of fusion constructs in cell lines deficient in different components of the class I loading pathway. The objectives of this work are: to determine whether folding of the β_2m-HC heterodimer precludes binding of calnexin, to see if tapasin and TAP are important for the recruitment of β_2m to heavy chain and for the expression of stable β_2m-HC heterodimers, and to show whether peptide binding to class I triggers release from the peptide-loading complex.

5.2 Methods

The methods used in this chapter are all detailed in chapter 2 and include: immunoprecipitation, western blotting (section 2.3), flow cytometry (section 2.2) and T cell killing assays (section 2.5.3).

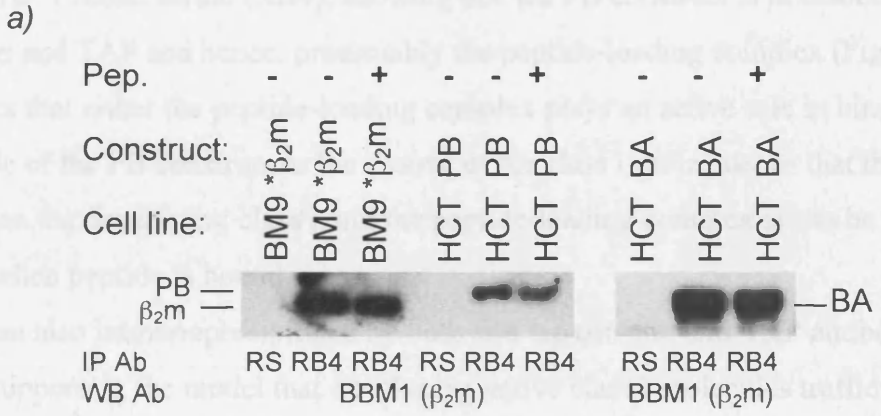
5.3 Results

5.3.1 Peptide binding does not automatically release class I from the PLC.

The main function of the peptide-loading complex is thought to be binding and optimisation of peptide to the maturing MHC class I molecule (Howarth et al., 2004;Zarling et al., 2003). Reports have been made that import of peptides into the ER by TAP (Knittler et al., 1999) or provision of high affinity peptide, cause the dissociation of class I from the peptide-loading complex (Suh et al., 1994). This would provide a quality control mechanism for peptide loading, in which it is envisaged that class I molecules are retained by the peptide-loading complex only until high affinity peptide is bound. In this model, the action of tapasin is somewhat similar to the role of HLA-DM in class II maturation.

To try and clarify whether provision of high affinity peptide causes dissociation of human class I from the peptide-loading complex, two different approaches were taken. Firstly, cells were permeabalised with 0.003% digitonin, provided with ATP and HLA-A2 binding peptide (as in Suh et al., 1994) and then immunoprecipitated with RB4 (anti-TAP). Secondly, cell lysates were incubated with peptide prior to immunoprecipitation (as in Knittler et al., 1999). However, neither method of peptide provision had any detectable affect on the association of β_2m, PB or BA with TAP, indicating that high affinity peptide did not lead to rapid dissociation of class I from TAP (Fig. 5.1).

Figure 5.1. Provision of high affinity peptide does not significantly alter the association between class I and the peptide-loading complex.
*a) 5x10^6 BM9 T cells or HCT cells expressing class I fusion proteins were permeabalised by incubation on ice with 50μl 0.003% digitonin for 7 minutes. Digitonin was diluted out with 450μl of cold PBS and permeabalised cells were incubated with ATP and HLA-A2 binding peptide GILGFVFTL for 20 minutes at 37°C. Cells were washed three times in cold PBS, lysed in 1% digitonin and immunoprecipitated with anti-TAP (RB4) or control (RS) rabbit serum. Eluted proteins were analysed by western blot with BBM.1 antibody. b) Cells were lysed in 1% digitonin and incubated with peptide for 1 hour at 4°C. Immunoprecipitation and western blot were performed as in (a). * Indicates native expression of protein*

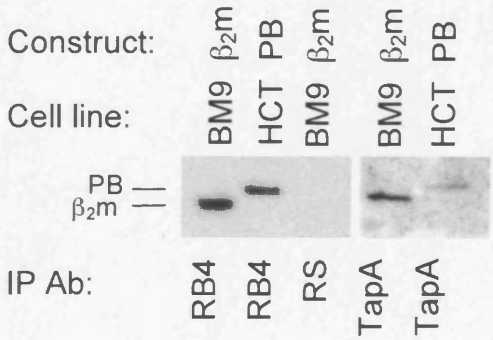


Although HLA-A2 binding peptide failed to trigger the release of class I from the peptide-loading complex in both these peptide release assays, care should be taken in drawing conclusions from these 'negative' results. The peptide release assay in figure 5.1a suffers from the fact that it is difficult to prove that the cells have been successfully permeabalised by treatment with 0.003% digitonin. One possible way to test this would be to incubate the permeabalised cells with fluorescently labelled peptide and then to analyse loading by flow cytometry. The experiment in figure 5.1b does not involve permeabalisation and the peptide must therefore have access to class I molecules in

the peptide-loading complex. However, since peptide provision occurs in the cell lysate the conditions are somewhat artificial. Because of the technical difficulties of the peptide release assays shown in figure 5.1 the PB fusion protein is a useful tool in dissecting the problem. Since the PB (peptide linked β_2m) construct already contains peptide, immunoprecipitation was used to check whether PB still traffics via the peptide-loading complex. In this assay the peptide must have access to the groove of the heavy chain of class I since it is covalently linked to β_2m. Interestingly it was found that the PB construct immunoprecipitated with both anti tapasin rabbit serum (Giles) and anti TAP 1 rabbit serum (RB4), showing that the PB construct is in association with tapasin and TAP and hence, presumably the peptide-loading complex (Fig. 5.2). This implies that either the peptide-loading complex plays an active role in binding the peptide of the PB construct to the groove of the class I molecule, or that the interaction between the developing class I and the peptide-loading complex is can be maintained even when peptide is bound.

β_2m can also immunoprecipitated by both anti tapasin and anti TAP antibodies (Fig. 5.2), supporting the model that developing native class I molecules traffic via the peptide-loading complex. Expression of β_2m in HCT cells was consistently low and immunoprecipitation using this cell line proved difficult. However, β_2m could be consistently immunoprecipitated from B cell lines such as BM9.

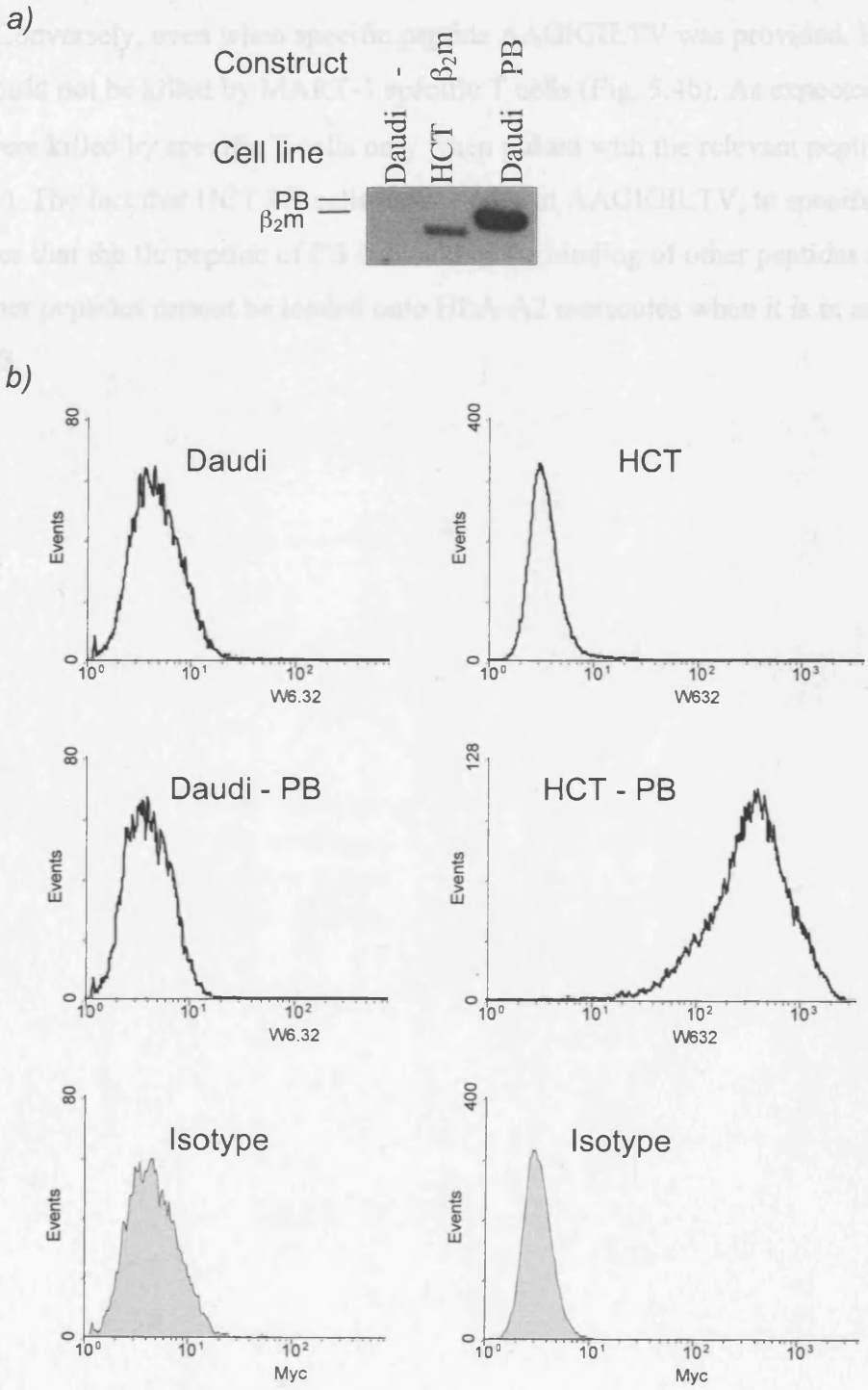
Figure 5.2. PB construct is found in association with the PLC. *Digitonin lysates from 10^7 BM9 cells or HCT cells expressing the class I fusion protein PB were immunoprecipitated with anti-TAP (RB4), anti-tapasin (Tap A) or control (RS) rabbit serum as indicated. Proteins were eluted with 2xSDS loading buffer separated by SDS-PAGE and immunoblotted with BBM.1 (mAb specific for human β_2m).*



It was shown in figure 5.2 that the peptide of the PB construct did not stop it from incorporating into the peptide-loading complex. However, it remains possible that the PB construct is found in the peptide-loading complex either because it interacts with other alleles of class I to which the flu peptide cannot bind, or because even once associated with PB, HLA-A2 traffics to the peptide-loading complex to load with another peptide. In order to try and exclude these possibilities two further experiments were performed. To test the interaction of the PB construct with other class I alleles, PB was introduced into the Daudi cell line, whose genotype like HCT is deficient in β_2m but in contrast to HCT does not contain HLA-A2 (Browning et al., 1995). Daudi cells were transduced with lentivirus expressing PB and protein expression was confirmed by western blot (Fig. 5.3a). Cell surface expression of class I was then assessed in transduced Daudi cells. However, there was no recovery of human class I expression in Daudi cells after introduction of PB (Fig. 5.3b), implying that it does not fold properly with the alleles of class I present in these cells.

Use of the HCT cell line transduced with PB provides a positive control for the virus, however, it does not prove the competency of the Daudi cell for class I expression. Although this has been demonstrated previously in the literature, it would have been beneficial to demonstrate recovery of class I expression in the Daudi cell by provision of β_2m alone.

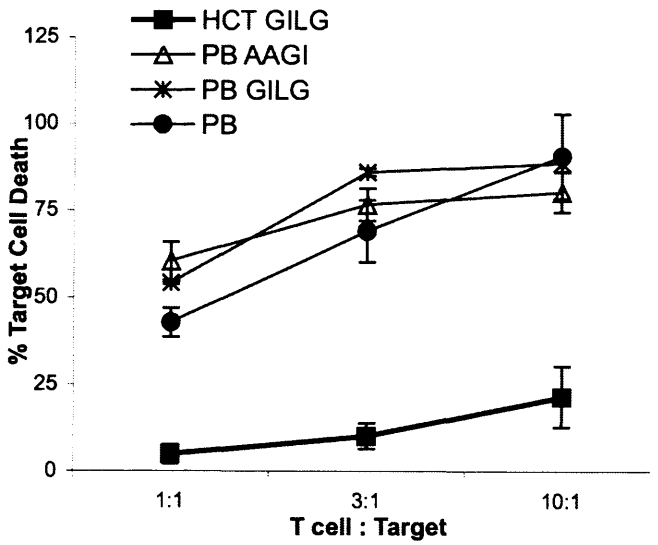
Figure 5.3. PB cannot pair up with class I alleles other than HLA-A2. *Daudi (β_2m -/-, HLA-A2 -/-) cells were transduced with lentivirus expressing PB. a) HCT β_2m cells, Daudi cells and Daudi cells transduced with PB virus, were analysed by western blot with BBM.1 antibody. b) HCT cells, HCT PB cells, Daudi cells and Daudi cells transduced with PB virus were analysed for cell surface expression of class I by flow cytometry, using W6.32 antibody.*



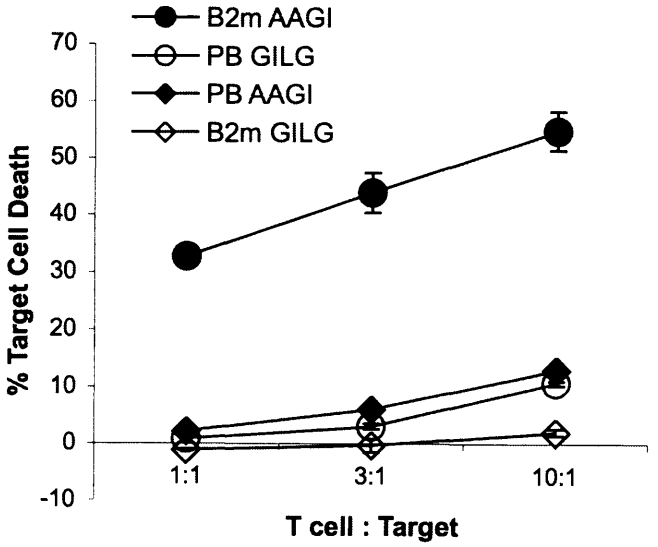
To test whether HLA-A2 can become loaded with an alternative peptide when associated with PB, HCT PB cells were pulsed with different peptides, and incubated with specific T cells. Whether HCT PB cells were provided with no peptide, flu peptide GILGFVFTL (GILG) or the MART-1 derived HLA-A2 restricted peptide AAGIGILTV (AAGI) (Coulie et al., 1994), they were killed effectively by flu specific T cells (Fig. 5.4a). Conversely, even when specific peptide AAGIGILTV was provided, HCT PB cells could not be killed by MART-1 specific T cells (Fig. 5.4b). As expected HCT β_2m cells were killed by specific T cells only when pulsed with the relevant peptide (Fig. 5.4 a and b). The fact that HCT PB cells cannot present AAGIGILTV, to specific T cells indicates that the flu peptide of PB is blocking the binding of other peptides and implies that other peptides cannot be loaded onto HLA-A2 molecules when it is in association with PB.

Figure 5.4. HLA-A2 cannot bind free peptide when associated with PB. *Target cells (HCT cells or HCT cells expressing β_2m or PB) were labelled with Cr^{51} and pulsed with flu peptide (GILG) or MART-1 peptide (AAGI) for 1 hour. After washing thoroughly target cells were mixed with flu specific T cells (a) or MART-1 specific T cells (b). Experiments were performed in triplicate and repeated 3 times. Data shown is mean and standard deviation of a representative experiment.*

a)



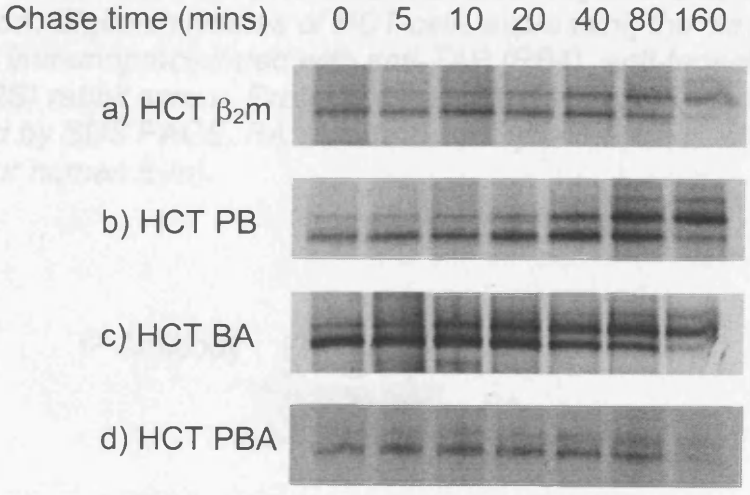
b)



5.3.2 The trafficking rate of the partially assembled class I constructs PB and BA is unaltered

Since the fusion proteins PB and BA are already effectively partially assembled class I molecules, their trafficking rate may be faster than normal class I molecules. To test this, pulse chase was performed by Dr. B Gao (Institute of Molecular Medicine, Oxford University) to measure the rate of ER export of these constructs. The results are included here as it is highly relevant to this work (Fig. 5.5). It was shown that the trafficking rate of neither the PB nor the BA construct to the Golgi body was accelerated. For heavy chain molecules in both HCT β_2m and HCT PB and for the linked chain molecule in HCT BA cells, 50% of molecules were Endo H resistant 60 mins after labelling and almost all detectable molecules were endo H resistant after 160 mins. In contrast the PBA construct did not become endo H resistant and by 160 minutes after labelling it had largely disappeared.

Figure 5.5. The export rate of class I molecules is similar in HCT cells expressing β_2m, PB and BA constructs. *HCT cells expressing β_2m, PB, BA or PBA were labelled with S^{35} met and cys for 10 minutes. Cells were 'chased' for up to 160 minutes lysed with Triton X-100 and immunoprecipitated with BBM.1 (mAb anti- β_2m). Proteins were treated with endo H enzyme, separated by SDS-PAGE and transferred onto nitrocellulose membrane. Photographic film was exposed to the membrane for 4 days, allowing detection of radioactive proteins. The proteins shown are wild type heavy chain (a) and (b), or linked chain molecules BA (c) or PBA (d). (Upper band – endo H resistant, lower band endo H sensitive.)*



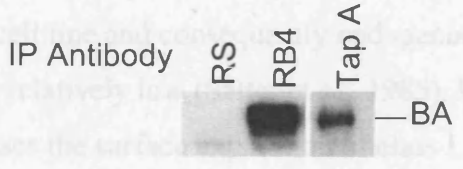
5.3.3 Tapasin and TAP do not play a crucial role in the formation of the class I β_2m-HC heterodimer.

The fusion proteins β_2m-A2 (BA) and β_2m-B44 (BB) provide a tool for investigating the importance of β_2m recruitment in class I folding. It is thought that tapasin and TAP do not interact with class I molecules until heavy chain has folded with β_2m. If this is correct, then it is unlikely that either tapasin or TAP plays a role in the recruitment of β_2m to heavy chain.

Since β_2m-A2 (BA) and β_2m-B44 (BB) have a covalent link between β_2m and heavy chain, they may be more stable than wild type β_2m-HC heterodimers. Wild type β_2m-HC heterodimers are known to become more stable when assembled with peptide and this may act as a trigger for the release of class I from the peptide-loading complex. If peptide free β_2m-HC linked chain constructs are more stable than their wild type counterparts, then this might influence their binding of tapasin or other molecules involved in class I folding.

To assess the interactions made by the BA construct, lysates of HCT BA cells were immunoprecipitated with rabbit serum against either tapasin or TAP. After recovery with protein G, samples were washed thoroughly and eluted in 2xSDS loading buffer and analysed by SDS PAGE and immunoblotting with mouse mAb BBM.1. Both TAP and tapasin strongly immunoprecipitated BA, indicating that it interacts not only with calnexin immediately after synthesis, but also follows the normal route of class I trafficking via the peptide-loading complex (Fig. 5.6).

Figure 5.6. BA construct interacts with both tapasin and TAP during maturation. *Digitonin lysates of HCT cells expressing the class I fusion protein BA, were immunoprecipitated with anti-TAP (RB4), anti-tapasin (Tap A) or control (RS) rabbit serum. Proteins were eluted in 2xSDS loading buffer and separated by SDS PAGE. BA was detected by immunoblot with BBM.1 (mAb specific for human β_2m).*

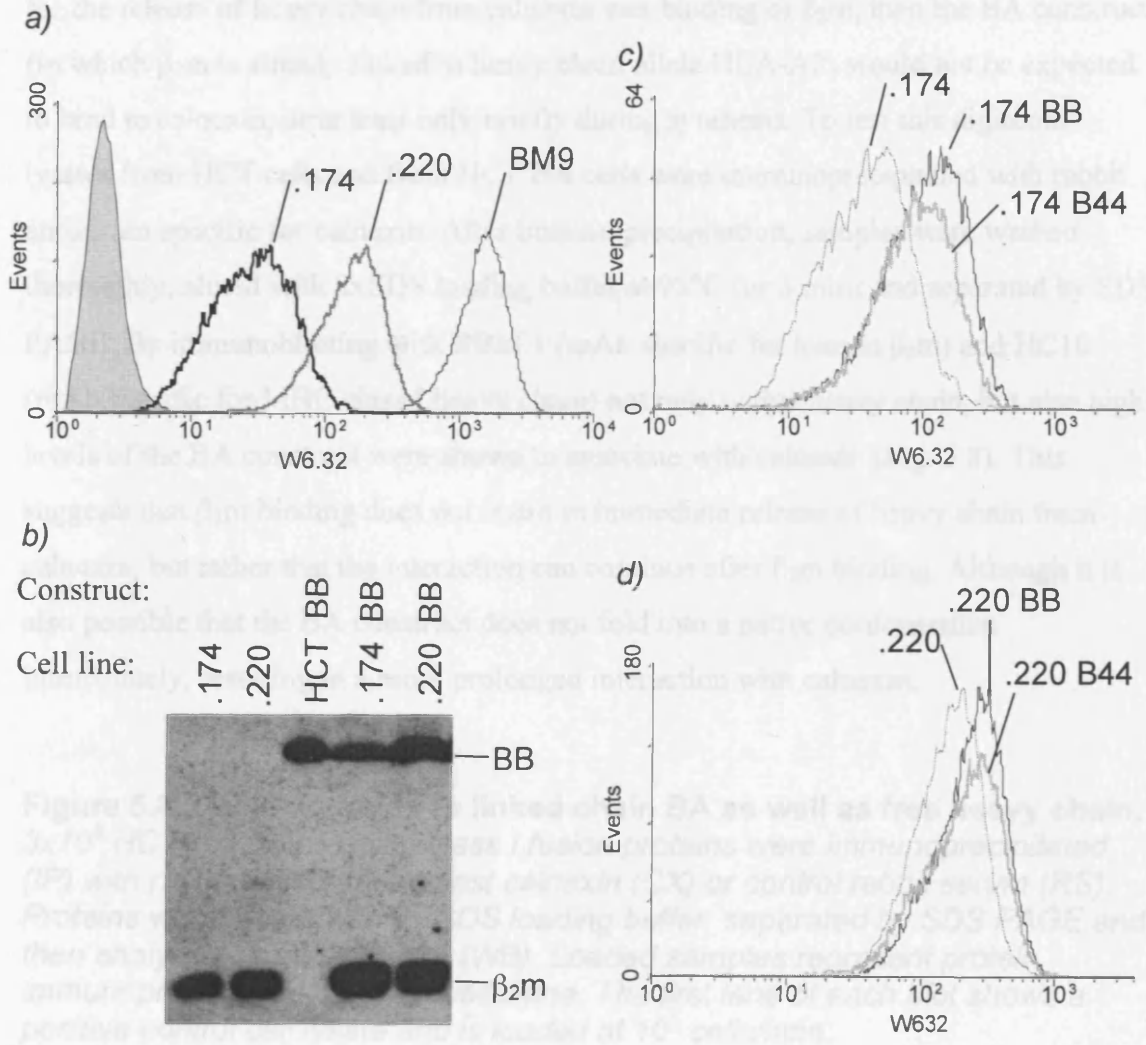


To assess the importance of the observed interaction between the peptide-loading complex and linked chain β_2m-heavy chain construct BA, lentivirus expressing the similar β_2m-B44 (BB) (see section 4.2) fusion construct was used to transduce tapasin deficient (.220) (Grandea, III et al., 1995) and TAP deficient (.174) (Cerundolo et al., 1990) human cell lines. As a control, lentivirus expressing HLA-B44 was also used to transduce these cells. HLA-B44 is a useful allele to use to assess the possible rescue of class I expression in these cells, since it is known to be particularly dependent on tapasin and the peptide-loading complex (Peh et al., 1998). In contrast HLA-A2 can be expressed well even in the absence of tapasin.

Expression of human class I is reduced in .174 and .220 cells in comparison to normal B or T cells and this can clearly be seen in a comparison of W6.32 staining of these cell lines (Fig. 5.7a). After transduction of .174 and .220 cells with lentivirus expressing BB, protein expression was confirmed by western blot using BBM.1 antibody (Fig. 5.7b). Detection of HLA-B44 by western blot is not possible since the cells already express other heavy chain alleles, which would interfere with the detection of the introduced protein. However virus preparations were tested for colony forming units in murine NIH.3T3 cells by selection with blasticidin and the titre of the 2 types of virus was shown to be similar (Table 4.3). Expression of β_2m-B44 did not enhance expression of surface class I on either .174 or .220 cells to a greater extent than HLA-B44 (Fig. 5.7 c and d). This indicates that not only do β_2m-HC molecules interact with and tapasin and the PLC, but also that this interaction is necessary for proper peptide loading and stable cell surface expression.
It can also be confirmed that neither tapasin of TAP is essential for the assembly of the β_2m-HC heterodimer, since linkage of β_2m to heavy chain fails to improve class I expression in either tapasin or TAP deficient cell lines.

A significant shift is seen when either HLA-B44 alone or the linked chain construct is expressed in .174 cells. This may reflect the fact that large parts of the MHC are missing in this cell line and consequently endogenously expressed levels of class I heavy chain are relatively low (Salter et al., 1985). Introduction of any heavy chain therefore increases the surface expression of class I. Alternatively, it is possible that the lentivirus itself has somehow caused a shift in the surface level of class I, although there is no reason to expect that the virus would cause a shift in class I expression.

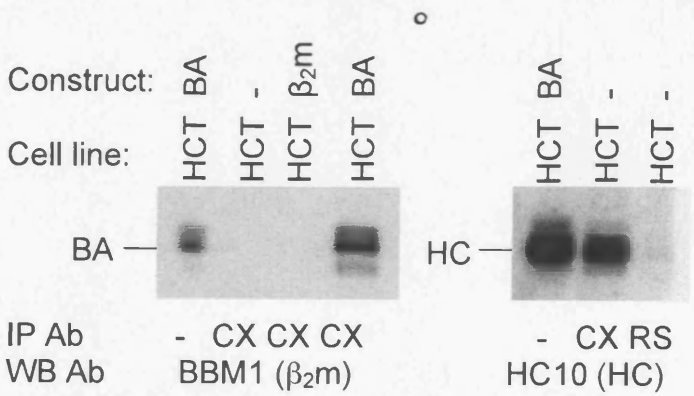
Figure 5.7. Linkage with β_2m does not enhance expression of HLA-B44 in the absence of tapasin or TAP.
a) Expression of class I on .174, .220 and BM9 cells was assessed by flow cytometry with W6.32 antibody. b) .174 and .220 cells were transduced with lentivirus expressing β_2m-B44 (BB). Expression of BB was analysed by immunoblot with BBM.1 antibody. c and d) .174 cells (c) and .220 cells (d) transduced with lentivirus expressing either HLA-B44 or BB, were analysed by flow cytometry as in (a).



5.3.4 Calnexin can associate with heavy chain alone or β_2m linked heavy chain (BA).

In human cells it has been shown that heavy chain binds to calnexin early after synthesis (Degen and Williams, 1991). However, when heavy chain is bound to β_2m, calnexin is thought to be replaced by calreticulin (Sadasivan et al., 1996). If the trigger for the release of heavy chain from calnexin was binding of β_2m, then the BA construct (in which β_2m is already linked to heavy chain allele HLA-A2) would not be expected to bind to calnexin, or at least only briefly during synthesis. To test this digitonin lysates from HCT cells and from HCT BA cells were immunoprecipitated with rabbit antiserum specific for calnexin. After immunoprecipitation, samples were washed thoroughly, eluted with 2xSDS loading buffer at 95°C for 5 mins and separated by SDS PAGE. By immunoblotting with BBM.1 (mAb specific for human β_2m) and HC10 (mAb specific for MHC class I heavy chain) not only native heavy chain, but also high levels of the BA construct were shown to associate with calnexin (Fig. 5.8). This suggests that β_2m binding does not result in immediate release of heavy chain from calnexin, but rather that the interaction can continue after β_2m binding. Although it is also possible that the BA construct does not fold into a native conformation immediately, resulting in a more prolonged interaction with calnexin.

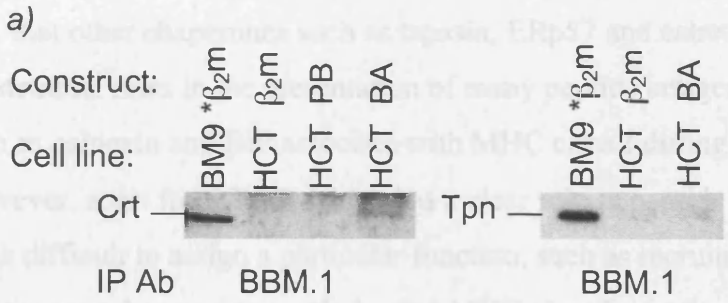
Figure 5.8. Calnexin binds to linked chain BA as well as free heavy chain. *$3x10^6$ HCT cells expressing class I fusion proteins were immunoprecipitated (IP) with rabbit antiserum against calnexin (CX) or control rabbit serum (RS). Proteins were eluted with 2x SDS loading buffer, separated by SDS PAGE and then analysed by western blot (WB). Loaded samples represent protein immunoprecipitated from 10^6 cells/lane. The first lane of each blot shows a positive control cell lysate and is loaded at 10^5 cells/lane.*

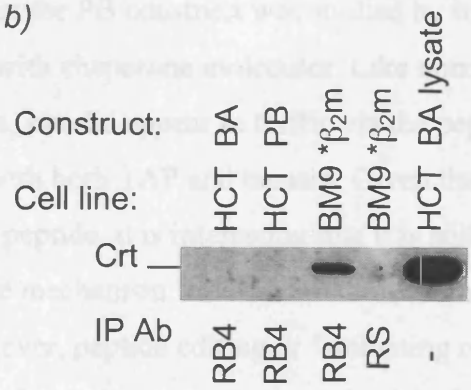


5.3.5 Immunoprecipitation using BBM.1 is not sensitive enough to verify the interactions made by fusion proteins in HCT cells.

In order to try and verify the interactions made by fusion proteins, immunoprecipitation of cells lysates was performed using BBM.1 (mouse mAb specific for human β_2m). Immunoprecipitates were analysed by immunoblotting with rabbit antisera specific for calreticulin, tapasin and ERp57. Unfortunately, it was not possible to detect any of these proteins by immunoblotting following BBM.1 immunoprecipitation of HCT cell lysates (Fig. 5.9a). However, it was possible to detect both calreticulin and tapasin in BBM.1 immunoprecipitates of BM9 B cells. This difference may reflect the fact that HCT cells are not immune cells and hence may express lower levels of class I heavy chain and class I machinery than BM9 cells. This hypothesis is supported by the fact that, calreticulin can also be detected in RB4 (anti-TAP) immunoprecipitates of BM9 cells but not those of HCT cells (Fig 5.9b). Since it was not possible to detect chaperone proteins in the BBM.1 immunoprecipitates any of the HCT cell lines it was not possible to double check the interactions so far indentified in this chapter using αCX, αTAP and αtapasin immunoprecipitation.

Figure 5.9. Calreticulin and tapasin can be detected in BBM.1 immunoprecipitates of BM9 cells but not HCT cell lines.
*a) Digitonin lysates of BM9 (B cells) or HCT cells expressing class I fusion proteins, were immunoprecipitated with BBM.1 (mAb specific for human β_2m) and purified by protein G beads. Proteins were eluted with 2xSDS loading buffer and separated by SDS PAGE. Calreticulin (Crt) and tapasin (Tpn) were detected by immunoblotting with the appropriate rabbit serum. b) Immunoprecipitation was performed with rabbit serum against TAP (RB4) or control rabbit serum (RS) as in (a) and calreticulin was detected by immunoblotting with Stressgene mouse anti-calreticulin mAb. *Indicates native expression of protein.*





5.4 Discussion

Antigen presentation to T cells involves a complex process of class I folding and peptide loading in the endoplasmic reticulum. MHC class I fusion proteins provide a unique way to investigate this process. Most existing work investigating MHC class I antigen processing, has inferred function to various chaperones through phenotypic variation in the ability of cells to process normal MHC class I molecules. In this way it has been possible to show that heavy chain, β_2m and TAP are vital to class I presentation, that other chaperones such as tapasin, ERp57 and calreticulin play important functional roles in the presentation of many peptide antigens, and that other proteins such as calnexin and BiP associate with MHC class I during the folding process. However, apart from TAP, which has a clear role in peptide provision to the ER it remains difficult to assign a particular function, such as recruitment of β_2m, peptide selection or release of correctly loaded MHC class I, to other chaperones within the complex.

In this chapter the PB construct was studied by immunoprecipitation to examine its interactions with chaperone molecules. Like normal β_2m, PB was not found to interact with calnexin, but did appear to traffic via the peptide-loading complex, showing association with both TAP and tapasin. Given that the PB construct already contains high affinity peptide, it is interesting that it is still traffics via the peptide-loading complex. The mechanism for class I release from the peptide-loading complex is not known. However, peptide editing or facilitating of peptide loading have been suggested as functions of tapasin; a proposed mechanism for this is the retention of empty class I molecules in the peptide-loading complex, followed by release upon binding of high affinity peptide. This would suggest that efficiently loaded class I molecules are generally not in contact with the peptide-loading complex. The fact that PB traffics via the peptide-loading complex does not support this model, but suggests that the interaction of class I and the peptide-loading complex occurs by default during processing and that the mechanism of release may be more subtle. Other evidence also suggests that a peptide-triggered release model may be too simplistic. Zarling et al. find that tapasin enhances the presentation of peptides whose binding affinity for class I is lower than those which bind in tapasin negative cells (Zarling et al., 2003). If binding of high affinity peptide causes release from the peptide-loading complex it seems unlikely that tapasin could enhance the loading of low affinity peptide in this way. Furthermore, Li et al. were able to isolate peptide loaded HLA-A2 class I molecules, which are

clearly still stably associated with both tapasin and TAP (Li et al., 2000). It seems likely then, that although there may be a mechanism for retaining empty class I molecules, this mechanism does not exclude the interaction of loaded class I molecules with the peptide-loading complex. The duration of this interaction may then be governed by the 'off rate' of class I from the complex and this may be affected by the nature of the peptide bound to class I. Peptides of much lower affinity would then have an opportunity to dissociate from class I before release from the PLC, allowing another round of peptide binding and resulting in the optimisation of peptide binding to class I molecules.

Others have proposed that the peptide-loading complex may act by holding class I molecules in a peptide receptive state and it is possible that the peptide-loading complex still acts on HLA-A2 in association with PB facilitating the binding of the peptide of the PB construct to the groove of HLA-A2. However, the failure of the MART-1 peptide to bind to HLA-A2 when it is associated with PB, shows that the binding of the peptide of the PB construct is highly dominant and suggests that it may bind to the groove of HLA-A2 before it reaches the peptide-loading complex where other peptides might be loaded.

Interestingly PB is unable to rescue class I expression in Daudi cells, which implies that the presence of the peptide prevents β_2m from interacting correctly with heavy chain alleles other than HLA-A2. Since the flu peptide is unlikely to bind the groove of other class I alleles it is possible that the free peptide is recognised as an 'unfolded protein' and the protein is degraded in the ER. If PB is unable to pair up with heavy chain alleles other than HLA-A2, and other peptides are unable to bind to HLA-A2 when it is in association with HLA-A2, this suggests that the peptide of PB is always resident in the peptide groove of HLA-A2 at the cell surface and that the relationship between HLA-A2 and PB is exclusive. Combined with evidence which shows that PB associates with both TAP and tapasin, this suggests that class I peptide loading does not automatically trigger release from the peptide-loading complex. This is furether supported by peptide release assays in which high affinity peptide fails to release class I molecules from the peptide-loading complex.

Immunoprecipitation with calnexin antibody provides a way of examining the 'early complex', which forms after protein synthesis. Interestingly the BA construct still associates with the chaperone calnexin, which is generally thought only to interact with free human heavy chain and not with β_2m-HC heterodimer. While some calnexin may

be bound to BA before it has folded into its native heterodimer conformation, the level of BA protein found in association with calnexin is surprisingly high. It is possible that BA does not fold efficiently into a heterodimer, and is therefore bound to calnexin for longer than expected, or that calnexin is involved in catalysing the formation of the heterodimer, and so is bound to BA just as it is bound to free heavy chain. In any case it is interesting that calnexin, which would not be expected to play any role in folding of the BA construct is found bound to it at high levels. A prolonged interaction of calnexin with class I is not unprecedented since in mouse cells calnexin is known to be associated with heavy chain in all stages of class I maturation in the ER (Suh et al., 1996). However, it is generally considered that mouse heavy chain alleles are able to maintain interactions with calnexin through extra N-glucosylation sites (Zhang and Salter, 1998). These sites are not present on human alleles or therefore the BA construct, so calnexin would not be expected to be able to interact with class I once it is bound to calreticulin and the peptide-loading complex. The role of calnexin in antigen processing remains obscure, since calnexin knockout cells exhibit an unaltered phenotype with respect to MHC class I (Scott and Dawson, 1995). In any case, the fact that BA traffics at the same rate as normal class I molecules suggests that its interaction with calnexin is not hindering processing.

The normal trafficking rate, coupled with the association of BA with calnexin and also tapasin and TAP, implies that the pathway for processing BA is similar to the processing of normal class I molecules. To test the reliance of linked chain β_2m-HC molecules on interactions with tapasin and TAP the BB construct was used since HLA-B*4402 is known to be particularly dependent on tapasin. However, it was shown that BB does not express at the cell surface any more strongly than HLA-B44 in these deficient cell lines implying that its expression is equally dependent on tapasin and TAP. Empty heterodimer molecules at the cell surface are common in tapasin deficient cells (Grandea, III et al., 2000) and the fact that β_2m-B44 is not expressed more highly at the cell surface of .220 cells than HLA-B44 implies that empty linked chain molecule is no more stable at the cell surface than wild type class I. Therefore, the mechanism for turnover of empty class I at the cell surface may not only be reliant on the dissociation of β_2m from heavy chain, but may also be sensitive to the peptide state of the class I heterodimer.

In this chapter it has been shown that calnexin binds to a preformed β_2m-HC class I heterodimer (BA) as well as to free heavy chain. This may be because the BA construct is slow to fold into its native conformation, but could also indicate that wild type heavy chain during synthesis and early folding and then dissociate spontaneously from calnexin. Once released from calnexin heavy chain may rapidly bind to calreticulin and β_2m, explaining the observed patterns of binding previously observed (Farmery et al., 2000;Sadasivan et al., 1996). Under this model the linked chain would be found to interact with calnexin after synthesis in the same way as free heavy chain, consistent with the findings in this chapter.

Work presented in this chapter has also shown that preformed β_2m-HC class I molecules interact with and are dependent on the peptide-loading complex. This implies that tapasin and TAP are not key to the recruitment of β_2m to heavy chain. If either component was crucial to the formation of the β_2m-HC heterodimer, then the linked chain molecule β_2m-B44 might be expected to express more effectively than separately expressed HLA-B44 and native β_2m in .174 (TAP deficient) or .220 (tapasin deficient) cells. Such a phenomenon was not observed. However, some caution should be taken when interpreting this negative result, since it is possible that TAP and tapasin facilitate class I antigen processing by more than one mechanism. Although linking β_2m to heavy chain removes one step in the folding class I, it would not compensate for other possible functions of tapasin or TAP. Since TAP is known to be crucial in peptide supply and tapasin is thought to play an important role in peptide loading, it is perhaps not surprising that linkage of β_2m to heavy chain does not rescue class I expression in these deficient cell lines.

Most interestingly it has been shown that peptide binding to class I does not necessarily trigger its release from the peptide-loading complex. This contradicts models of peptide loading which rely on peptide mediated release of class I from the peptide-loading complex. Instead the results support a role for the peptide-loading complex in the facilitating of class I loading, by providing a suitable environment for loading to take place.

In conclusion, it is found that partially preformed class I molecules interact with the same chaperone molecules as wild type class I. Supporting the hypothesis that the interactions of the developing class I molecule follow a 'default' pathway involving calnexin and the peptide-loading complex. Although it is possible that some components of processing may become redundant in the process of folding linked chain molecules, the basic pathway for class I maturation remains the same. Chaperones

molecules should therefore be seen as 'passive catalysts' in the process of class I folding.

6 An allele specific molecule is implicated in the surface expression of β_2m linked HLA-B*4402

6.1 Introduction

It is well documented that although expression of mouse MHC class I Heavy Chain (HC) in human cells results in cell surface expression of mouse class I (Alexander et al., 1989), heavy chain alleles of human MHC class I often express poorly in murine cells (Peh et al., 1998). Although, HLA-A2 is able to express stably in normal murine cells (Irwin et al., 1989) or in Daudi (human B cells, that are deficient for β_2m) cells provided with mouse β_2m (Seong et al., 1988), expression of alleles such as HLA-B8 and HLA-A25 is enhanced by the provision of human β_2m (Wang et al., 1993). For other alleles, such as HLA-B*4402, cell surface expression in mouse cell lines remains reduced even in the presence of human β_2m and full recovery requires co-expression of human tapasin (Peh et al., 1998). It is possible to exploit this species incompatibility to study antigen processing; effectively with respect to HLA-B44 the K42 (crt -/-) murine cell line represents a doubly deficient cell line, lacking both calreticulin and human tapasin. The K41 cell line on the other hand is a proficient in calreticulin but still lacking human tapasin. It is widely believed that chaperone molecules of MHC class I antigen processing work cooperatively; in other words a combination of interactions between proteins stabilises the peptide-loading complex for efficient peptide loading. Because of the high dependence of HLA-B44 on human tapasin the cooperative effects of chaperones in class I antigen processing can be investigated by expression in mouse K41 and K42 cells.

For the purposes of this work the linked chain molecule β_2m-B44 has been expressed in both K41 and K42 (crt -/-) cells using a lentivirus system (section 4.3.2). The rationale for the use of this linked chain molecule is two-fold. Firstly, it provides human β_2m to the cells, which is required for the expression of HLA-B44 in murine cells. Secondly, because β_2m is covalently linked to HLA-B44 the β_2m-B44 heterodimer it is already partially assembled and may have altered requirements for either human tapasin or for the chaperone calreticulin. It is known from the murine RMA-S (TAP -/-) cell line, that empty or 'low affinity peptide' bound class I molecules can traffic to the cell surface if their stability is increased by cooling the cells to 20-26°C (Ljunggren et al., 1990;De Silva et al., 1999). A similar situation could be envisaged for the linked chain constructs; if they are more stable than separately expressed class I molecules they may traffic to the cell surface better without optimisation of peptide by tapasin. For purposes

of comparison viruses expressing HLA-B44 and β_2m were also created (section 4.3) and a linked chain protein for the allele HLA-A2 (β_2m-A2) has also been used. Although the usual trafficking of the MHC class I allele HLA-A2 involves interactions with TAP and tapasin, it is widely documented to be able to traffic independently of this pathway (Lewis et al., 1998). As a result it expresses stably in tapasin deficient human cell lines and also in mouse cells. One would therefore expect HLA-A2 to express well in K41 cells, but since its calreticulin dependence is not known, how it will express in K42 cells is not clear.

K42 is an immortalised calreticulin deficient mouse fibroblast cell line (Mesaeli et al., 1999), which displays decreased surface expression of MHC class I in comparison to the calreticulin proficient equivalent cell line K41 (Gao et al., 2002). The calreticulin dependent expression level of class I, and the simultaneous association of calreticulin and β_2m with heavy chain, has led to speculation that calreticulin may be crucially involved in the formation and maintenance of the β_2m-HC heterodimer. Since linked chain molecules already express both components of the β_2m-HC heterodimer, there should be no requirement for β_2m recruitment. By comparing the expression level of both β_2m-B44 and β_2m-A2 in K41 cells with the level in K42 (crt -/-) cells an assessment of the importance of calreticulin in β_2m recruitment may be possible. The expression of human class I constructs in murine cell lines will also add to the body of knowledge of species specific differences in class I processing between mice and humans.

6.2 Methods

Cell lines K41 β_2m-B44 and K42 β_2m-B44 were used as established in section 4.3.2. K41 β_2m-B44 was further subcloned by limited dilution cloning to obtain a pure population. Other methods used in this chapter are all detailed in chapter 2 and include: western blotting (section 2.3), transfection (section 2.1.10), flow cytometry (section 2.2) and T cell killing assays (section 2.5.3).

6.3 *Results*

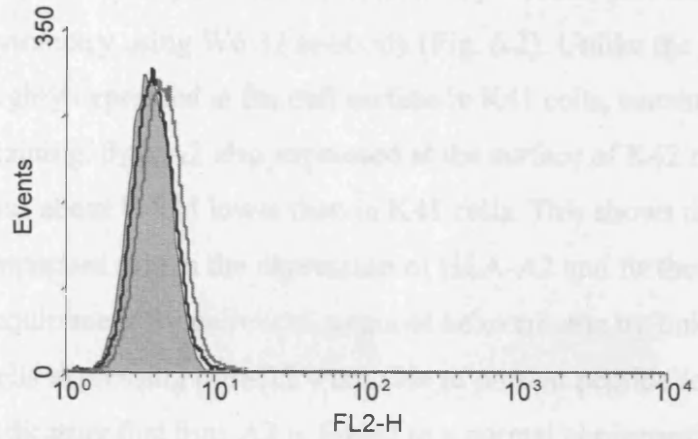
6.3.1 β_2m-B44 fails to express at the cell surface of murine K41 and K42 cells.

K41 and K42 cells were transduced with virus expressing β_2m-B44 and selected with blasticidin. Surviving colonies were expanded and assessed for expression of β_2m-B44 by western blot and positive clones were chosen (section 4.3.3). The phenotype of these cells was assessed by flow cytometry using BBM.1 or W6.32 antibody, which recognise human β_2m and folded human class I respectively and do not cross react with mouse class I. Expression of HLA-B44 in murine L cells also expressing human β_2m has previously been shown to produce a 10-fold increase in surface staining using W6.32 antibody (Peh et al., 1998). Therefore, it was expected that murine K41 cells endogenously expressing β_2m-B44 would stain with W6.32 and that K42 (calreticulin deficient murine) cells expressing β_2m-B44 might stain somewhat less strongly. However, as shown in figure 6.1, endogenous expression of β_2m-B44 caused no detectable shift in W6.32 staining in either K42 or K41 cells. To confirm the absence of cell surface expression of β_2m-B44 in transduced cell lines, cells were stained with BBM.1 antibody, which recognises human β_2m regardless of the folding status of class I. However, like W6.32 antibody, BBM.1 did not stain K41 or K42 cells expressing β_2m-B44, providing further evidence that despite strong expression of protein, detected by western blot using BBM.1 antibody (Fig. 4.11), there is no cell surface expression of the linked chain construct in these cells (Fig. 6.1).

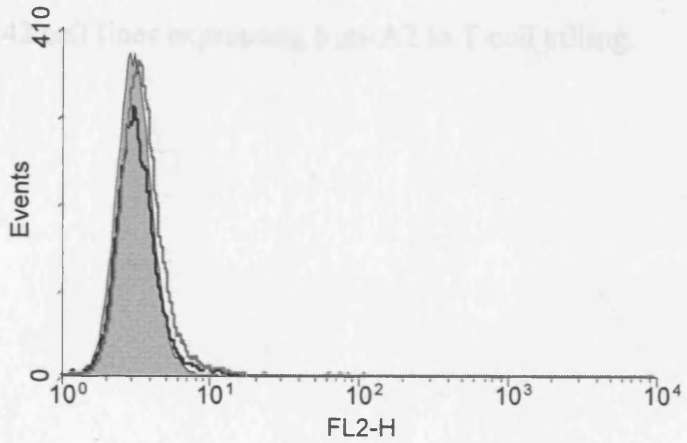
Figure 6.1. β_2m-B44 fails to express at the cell surface of K41 and K42 cells.

K41 cells (a) and K42 cells (b) transduced with 'pLenti β_2m-B44-V5', and stably selected with blasticidin, were stained for 1 hour with primary antibody W6.32 (black line), BBM.1 (grey line) or isotype control (shaded histogram). Cells were washed and stained for 1 hour with PE labelled secondary antibody and analysed by flow cytometry.

a)



b)

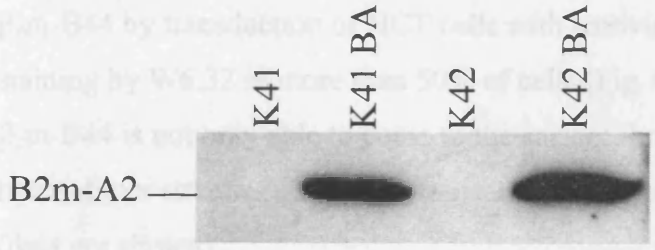


6.3.2 BA construct is able to express normally in K41 cells and at a reduced level in K42 cells.

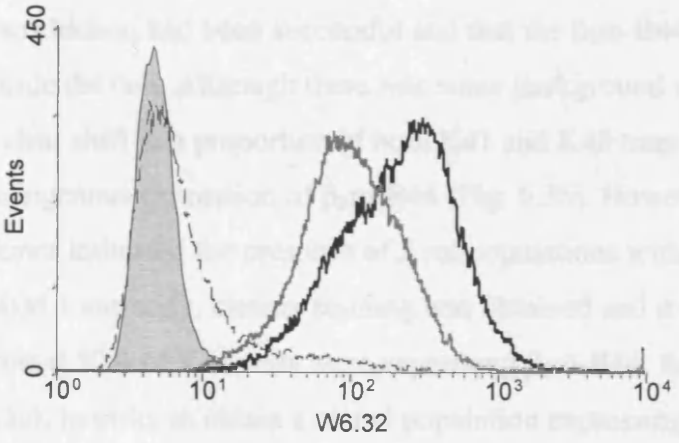
To check that there is no other defect in K41 and K42 cells which inhibits expression of human class I, a linked chain construct for HLA-A2 was expressed in these cells. β_2m-A2 was introduced by transfection, and cells were selected using zeocin (50μg/ml). Expression of β_2m-A2 was detected by western blot and cell surface expression by flow cytometry using W6.32 antibody (Fig. 6.2). Unlike the β_2m-B44 construct, β_2m-A2 was highly expressed at the cell surface in K41 cells, causing a 2-log shift in W6.32 staining. β_2m-A2 also expressed at the surface of K42 cells and interestingly this shift was about 8-fold lower than in K41 cells. This shows that calreticulin plays an important role in the expression of HLA-A2 and furthermore indicates that the requirement for calreticulin cannot be overcome by linkage of HLA-A2 to β_2m. K42 cells expressing β_2m-A2 were able to present peptide to CTL in a killing assay, indicating that β_2m-A2 is folded in a normal conformation at the cell surface of these murine cells (Fig. 6.2c). Unfortunately, expression of β_2m-A2 in the K41 cell line was lost after 2-3 passages, so it was not possible to compare the susceptibility of K41 and K42 cell lines expressing β_2m-A2 to T cell killing.

Figure 6.2. β_2m-A2 expresses in K41 and K42 cells and can present antigen to CTL. *K41 and K42 cells were transfected with β_2m-A2 and stably selected with zeocin. a) Cells were assessed by western blot for expression of β_2m-A2 using mAb BBM.1. b) K41 cells (thin black line) and K41 β_2m-A2 cells (thick black line) and K42 β_2m-A2 cells (thick grey line), were assessed for surface human MHC class I by flow cytometry using mAb W6.32. (shaded histogram shows isotope control) c) K42 cells or K42 β_2m-A2 cells were pulsed with influenza matrix protein derived peptide (GILGFVFTL) and labelled with Cr^{51} for 1 hour. Cells were washed and incubated for 4 hours with CTL_{FLU}, a T cell clone recognising GILGFVFTL presented by HLA-A2. Cell killing was calculated from the release of chromium from labelled target cells, normalised using values for detergent mediated and spontaneous release.*

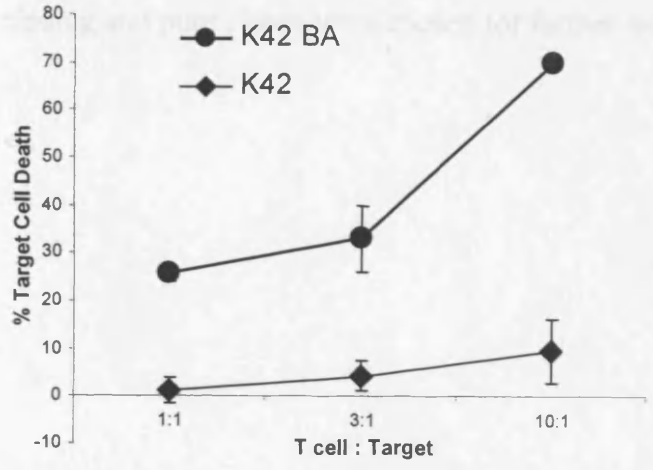
a)



b)



c)

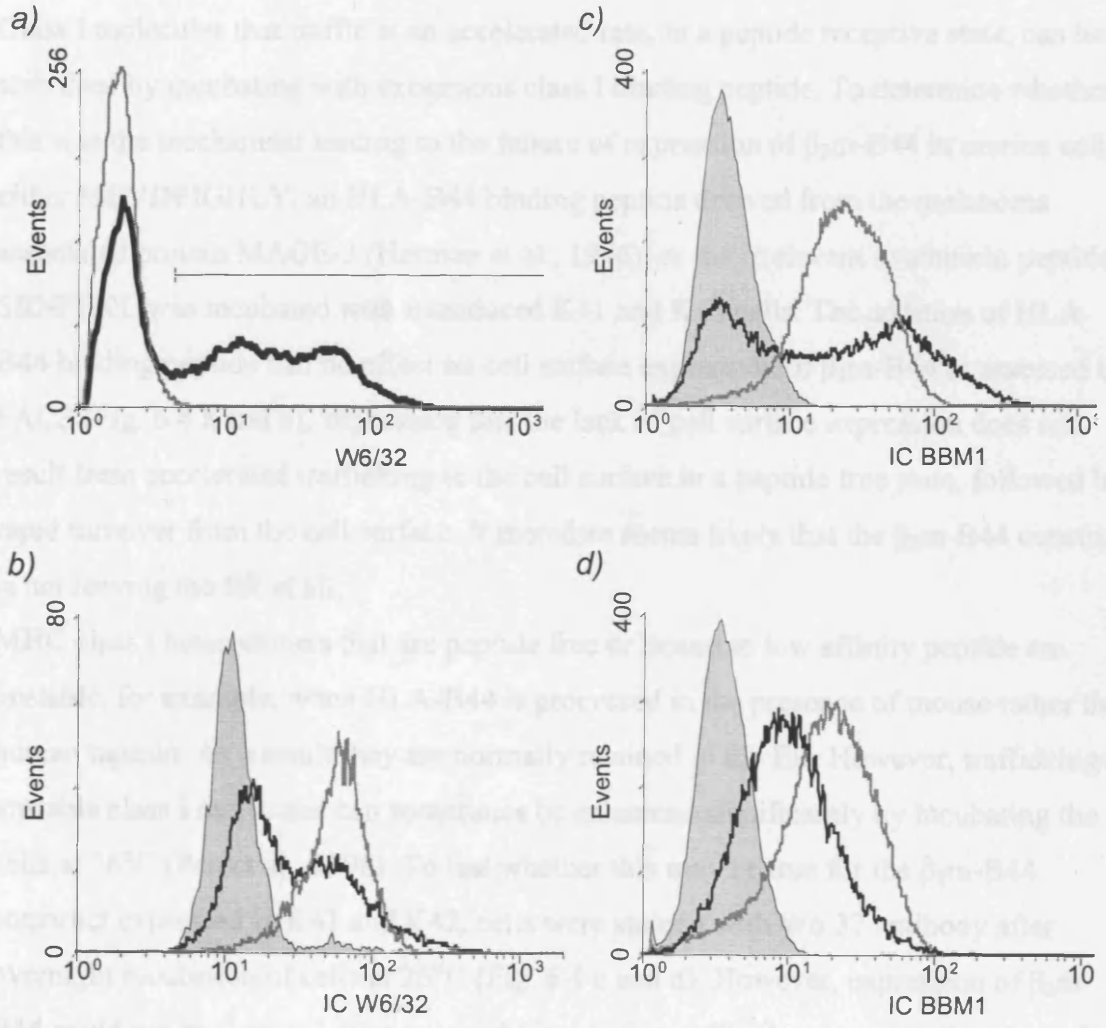


6.3.3 β_2m-B44 is able to fold into a native heterodimer structure inside murine cells and expresses normally on the surface of human cells.

To confirm that the β_2m-B44 molecule is able to fold correctly and that it can be recognised by both and W6.32 and BBM.1 antibodies two further experiments were performed. Firstly the β_2m-B44 construct was expressed in the human colon carcinoma cell line HCT, which is deficient in β_2m (Dexter et al., 1979), and secondly K41 and K42 cells transduced with β_2m-B44 were stained for intracellular protein expression and analysed by flow cytometry. Flow cytometry shows that transient expression of β_2m-B44 by transduction of HCT cells with lentivirus causes a significant shift in staining by W6.32 in more than 50% of cells (Fig. 6.3a). This shows that in human cells β_2m-B44 is not only able to come to the surface, but that it is also forms a normal heterodimer structure. Similarly transduced HCT cells can also be stained by BBM.1 (data not shown).

Intracellular staining of K41 and K42 cells transduced cells with W6.32 confirmed that transduction had been successful and that the β_2m-B44 construct was folding correctly inside the cell. Although there was some background using W6.32 antibody, there was a clear shift in a proportion of both K41 and K42 transduced cells, indicating the endogenous expression of β_2m-B44 (Fig. 6.3b). However, staining of K41 β_2m-B44 clones indicated the presence of 2 subpopulations within transduced cells. Using BBM.1 antibody, cleaner staining was obtained and it was confirmed that although around 50% of K41 cells were expressing β_2m-B44, the clones were not pure (Fig. 6.3c). In order to obtain a clonal population expressing β_2m-B44 in all cells, limited dilution cloning was performed. Expression was then checked by intracellular BBM.1 cloning and pure clones were chosen for further work (Fig. 6.3d).

Figure 6.3. The β_2m-B44 construct is able to express at the cell surface in human cells and is able to fold inside murine cells. *a) HCT cells (grey line) and HCT cells transduced with β_2m-B44 virus (black line) were stained for surface expression of human class I (mAb W6.32). M1=54.8 %. b and c) K41 β_2m-B44 (black line) and K42 β_2m-B44 (grey line) were permeabalised with 0.1% saponin and stained for intracellular expression of folded human class I (mAb W6.32) (b), or human β_2m (mAb BBM.1) (c). d) K41 β_2m-B44 cells were subcloned to obtain a pure population. Cells were then stained for intracellular human β_2m, as in (c). All shaded histograms show non transduced K41 cells.*



6.3.4 Absence of cell surface β_2m-B44 does not result from accelerated trafficking or instability of the construct.

Two main mechanisms have been reported that can explain impaired surface expression of MHC class I. Either class I molecules fail to traffic to the cell surface and instead are retained in the ER and degraded (Paulsson et al., 2001), or trafficking is accelerated and suboptimally loaded or unstable MHC class I is rapidly lost from the cell surface (Lewis et al., 1996).

Class I molecules that traffic at an accelerated rate, in a peptide receptive state, can be stabilised by incubating with exogenous class I binding peptide. To determine whether this was the mechanism leading to the failure of expression of β_2m-B44 in murine cells, either MEVDPIGHLY, an HLA-B44 binding peptide derived from the melanoma associated protein MAGE-3 (Herman et al., 1996), or the irrelevant ovalbumin peptide SIINFEKL was incubated with transduced K41 and K42 cells. The addition of HLA-B44 binding peptide had no effect on cell surface expression of β_2m-B44 as assessed by FACS (Fig. 6.4 a and b), suggesting that the lack of cell surface expression does not result from accelerated trafficking to the cell surface in a peptide free state, followed by rapid turnover from the cell surface. It therefore seems likely that the β_2m-B44 construct is not leaving the ER at all.

MHC class I heterodimers that are peptide free or bound to low affinity peptide are unstable, for example, when HLA-B44 is processed in the presence of mouse rather than human tapasin. As a result they are normally retained in the ER. However, trafficking of unstable class I molecules can sometimes be enhanced significantly by incubating the cells at 26°C (Peh et al., 1998). To test whether this may be true for the β_2m-B44 construct expressed in K41 and K42, cells were stained with W6.32 antibody after overnight incubation of cells at 26°C (Fig. 6.4 c and d). However, expression of β_2m-B44 could not be detected after overnight incubation at 26°C, suggesting that the lack of cell surface expression does not result from low stability of the β_2m-B44 structure and inability to load peptide in the ER.

A positive control using a cell line such as T2 would have been beneficial here to demonstrate that under certain conditions class I expression can be enhanced by provision of class I binding peptide or by incubating the cells at 26°C.

Figure 6.4. Incubation at 26°C or provision of peptide cannot restore cell surface expression of β_2m-B44. *a) and b), K41 and K42 cells stably expressing β_2m-B44, were incubated for 1 hour with HLA-B44 binding peptide MEVDPIGHLY or an irrelevant peptide SIINFEKL. c) and d) the same cells were incubated overnight at either 37°C or 26°C. All cells were stained for expression of human class I with W6.32 antibody. Bottom panel - staining of HEK 293 cells was used as a positive control and isotope contol staining is also shown (shaded).*

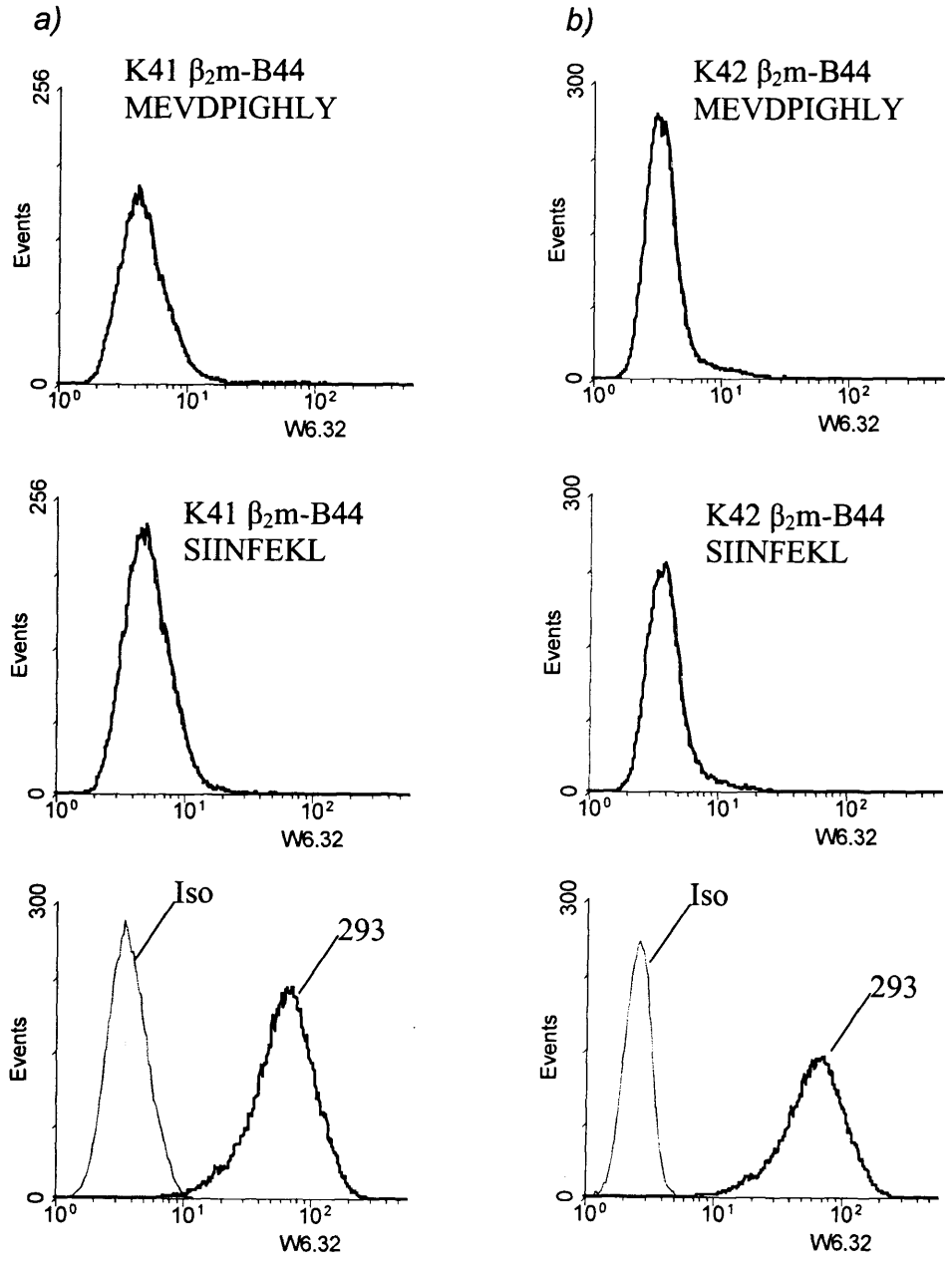
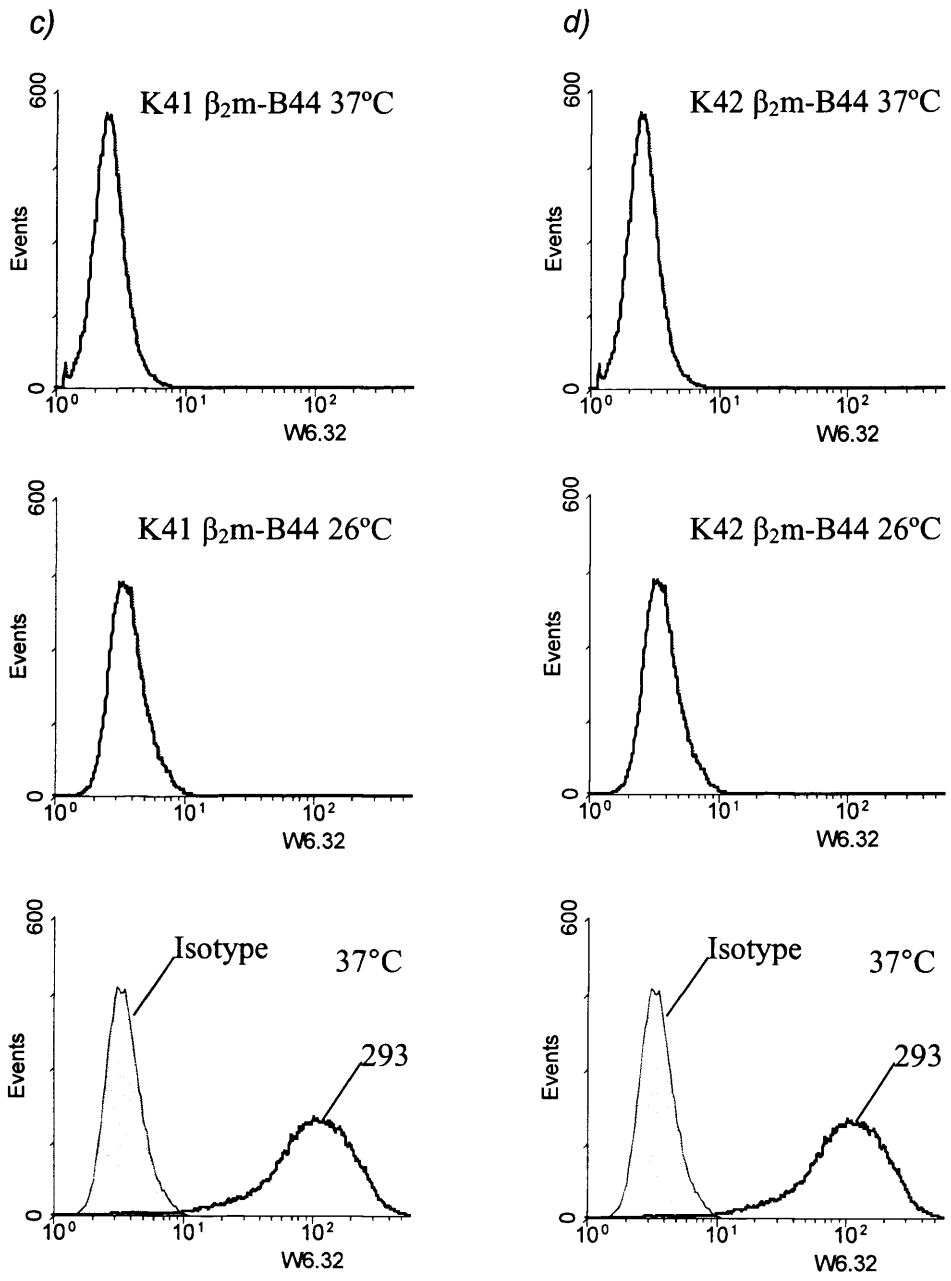


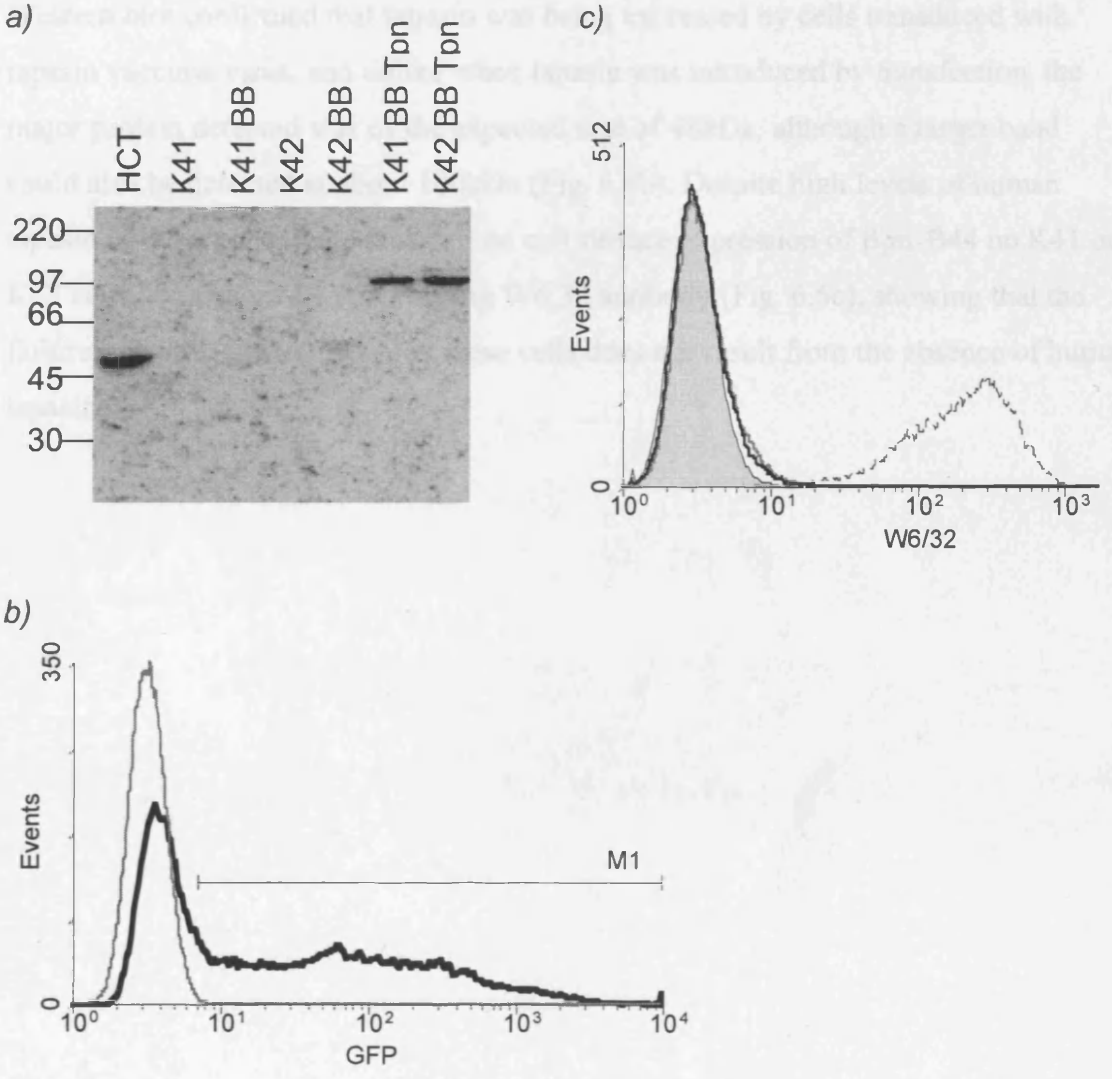
Figure 6.4 continued.



6.3.5 Provision of human tapasin does not facilitate surface expression of β_2m-B44

Since β_2m-A2 was able to express in murine K41 and K42 cells, the failure of β_2m-B44 to express must be an allele specific phenomenon. In light of previous studies showing a high dependency of HLA-B44 on human tapasin (Peh et al., 1998), K41 and K42 cells transduced with β_2m-B44 were transfected with plasmid expressing either native human tapasin or a soluble human tapasin molecule shown previously to be able to facilitate class I loading (Gao et al., 2004). 48 hours after transfection using lipofectamine 2000, cells were assessed for human tapasin expression by western blotting and for cell surface human MHC class I expression by flow cytometry. Cells transfected with tapasin, expressed a protein that could be detected by western blot using Giles anti-tapasin rabbit serum; however, the protein detected was larger than expected, around 100kDa rather than 48kDa (Fig. 6.5a). This band was only detectable in cells transfected with human tapasin and not in untransfected cells. Control human HCT cells expressed a protein, which stained with Giles and was of the expected size. The exact nature of the lower mobility band in transfected cells is not clear although it may represent a dimer of tapasin or alternatively, a stable complex of tapasin with Erp57. A stable disulfide bridge has been identified between ERp57 and tapasin (Peaper et al., 2005) and it may be that at this fairly low level of tapasin expression all the tapasin is associated with Erp57. GFP transfection indicated that around 50% of K41 and K42 cells were transfected by using lipofectamine 2000 (Fig. 6.5b). However, transfection with either tapasin or soluble tapasin did not increase the cell surface expression of human class I in either K41 or K42 cells endogenously expressing β_2m-B44 (Fig. 6.5c).

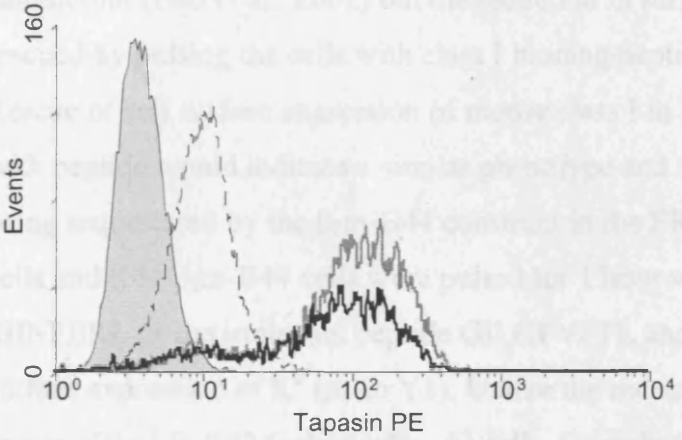
Figure 6.5. Transfection of human tapasin into K41/K42 β_2m-B44 cells has no effect on cell surface human MHC class I. a) *K41 β_2m-B44 (BB) and K42 β_2m-B44 (BB) cells were transfected with wild type tapasin (Tpn) using lipofectamine 2000. Expression of tapasin was detected by western blot using anti-tapasin rabbit serum Giles. b) K41 β_2m-B44 cells (grey line) and K41 β_2m-B44 cells transfected with GFP (black line) were analysed by flow cytometry for fluorescence in the FL-1 channel (GFP). M1=58%. c) Surface expression of human MHC class I on positive control HEK 293 cells (thin black), and on K41 β_2m-B44 cells transfected with tapasin (thick black) or soluble tapasin (thick grey) was assessed by flow cytometry using W6.32 antibody. Shaded histogram shows non transfected cells.*



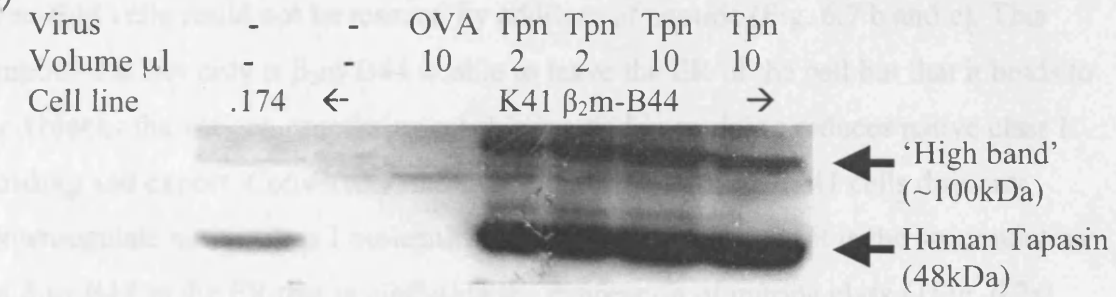
Since the transfected tapasin detected by western blot was not of the expected size, tapasin was also introduced into these cells using vaccinia virus, containing a human tapasin expression cassette. Vaccinia virus was titrated by intracellular FACS staining using Giles anti tapasin rabbit serum, and a vaccinia virus containing ovalbumin was used as a control. Over 90% of K41 cells expressed tapasin after transduction with 2ul of the human tapasin vaccinia virus preparation (Fig. 6.6a) and cell death assessed by trypan blue staining was less than 15% (data not shown). Increasing the viral load did not improve the efficiency of transduction and resulted in significant cell death. Western blot confirmed that tapasin was being expressed by cells transduced with tapasin vaccinia virus, and unlike when tapasin was introduced by transfection, the major protein detected was of the expected size of 48kDa, although a larger band could also be detected at about 100kDa (Fig. 6.6b). Despite high levels of human tapasin in these cells, there was still no cell surface expression of β_2m-B44 on K41 or K42 cells as assessed by FACS using W6.32 antibody (Fig. 6.6c), showing that the failure of β_2m-B44 expression in these cells does not result from the absence of human tapasin.

Figure 6.6. Expression of human tapasin by vaccinia virus does not allow expression of β_2m-B44 on K41 cells. a) *K41 β_2m-B44 cells transduced with 2μl (thick grey) or 10μl (thick black) of vaccinia virus expressing human tapasin (Tpn), or 10μl of vaccinia expressing irrelevant ovalbumin (OVA) protein (thin black), were analysed for intracellular expression of tapasin by permeabalisation with 0.1% saponin and staining with rabbit antiserum Giles specific for human tapasin. b) Human .174 B cells or cells as in (a) were analysed by western blot for expression of human tapasin using Giles antiserum. c) K41 β_2m-B44 cells transduced with 2μl of vaccinia virus expressing human tapasin (black) or ovalbumin (grey) were assessed by FACS for expression of human class I by staining with W6.32 Ab. Shaded FACS histograms show non transduced K41 β_2m-B44 cells.*

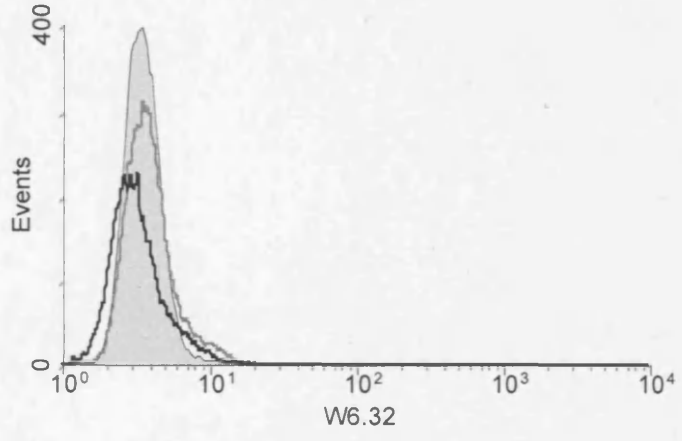
a)



b)



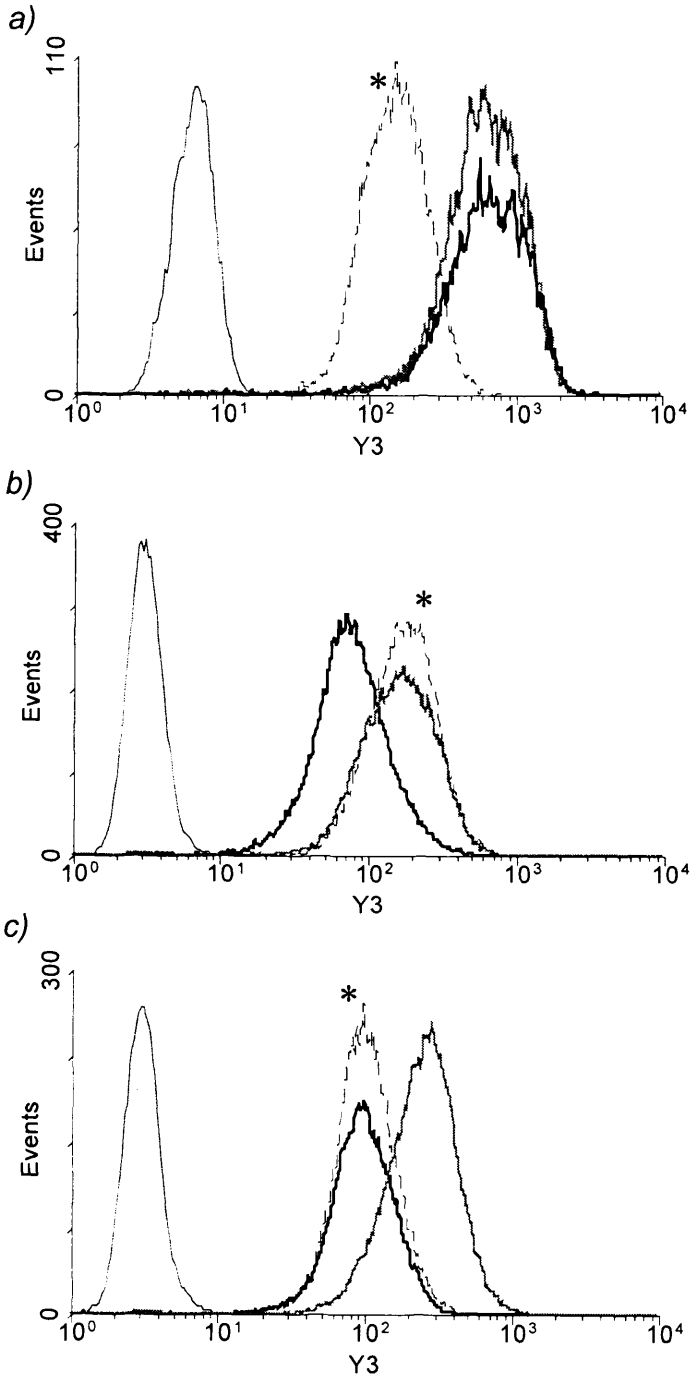
c)



6.3.6 B_2m-B44 blocks processing of native class I in K41 cells.

Given that β_2m-B44 is expressed at high levels in transfected cells but does not appear to leave the ER, it was reasoned that ER machinery might be affected in its ability to process other class I molecules. In order to test this, the level of mouse MHC class I on K41 cells expressing β_2m-B44 was measured by flow cytometry using mAb Y3. The expression of β_2m-B44 in K41 cells was found to lower the level of expression of native MHC class I by around 8-10 fold (Fig. 6.7a). K42 cells display a similar 10-fold reduction in class I expression in comparison to K41 cells because of the absence of calreticulin (Gao et al., 2002) but the reduction in surface class I in K42 cells can be rescued by pulsing the cells with class I binding peptide (Fu et al., 2006 under review). Rescue of cell surface expression of mouse class I in K41 β_2m-B44 cells by pulsing with peptide would indicate a similar phenotype and suggest that maybe calreticulin is being sequestered by the β_2m-B44 construct in the ER. To test this K41 cells, K42 cells and K41 β_2m-B44 cells were pulsed for 1 hour with H2-K^b binding peptide SIINFEKL or the irrelevant peptide GILGFVFTL and cells were stained for cell surface expression of K^b (mAb Y3). Unlike the reduction in surface expression of mouse class I in K42 (calreticulin -/-) cells, the reduction in K^b expression in K41 β_2m-B44 cells could not be rescued by addition of peptide (Fig. 6.7 b and c). This implies that not only is β_2m-B44 unable to leave the ER of the cell but that it binds to and blocks the antigen processing machinery, and in so doing reduces native class I loading and export. Conversely, the expression of β_2m-A2 in K41 cells does not downregulate native class I molecules supporting the view that it is the accumulation of β_2m-B44 in the ER that is inhibiting the expression of murine class I (Fig. 6.7a).

Figure 6.7. β_2m-B44 but not β_2m-A2 blocks the export of native class I to the cell surface. *All cells were assessed for surface expression of K^b by FACS using mAb Y3 (shaded histograms show isotype controls). Where indicated cells were incubated at 37°C for 1 hour with the irrelevant peptide GILGFVFTL or the K^b binding peptide SIINFEKL. a) K41 cells (thick grey) and K41 β_2m-B44 cells (thin black *) or K41 β_2m-A2 cells (thick black). b) K41 cells (thick grey), K42 cells with GILGFVFTL peptide (thick black) or SIINFEKL peptide (thin black *). c) K41 cells (thick grey), K41 β_2m-B44 with GILGFVFTL peptide (thick black) or SIINFEKL peptide (thin black *).*



6.4 Discussion

A detailed basic phenotype for the calreticulin deficient cell line K42 was first published in 2002 by Gao et al. and showed that class I expression was reduced, and that certain peptides were not properly presented to T cells in the absence of calreticulin (Gao et al., 2002). This was an important step to understanding the role of calreticulin in class I assembly, since as a general ER chaperone its interactions with class I, like those of calnexin (Scott and Dawson, 1995) could have proved to be redundant. However, the observed phenotype of the K42 (crt -/-) cell line for mouse class I molecules, highlights the importance of calreticulin in antigen processing.

Since it is known that the binding of calreticulin to heavy chain coincides with the binding of heavy chain to β_2m (Sadasivan et al., 1996), it has been suggested that the role of calreticulin might be to promote the binding of β_2m to heavy chain and subsequently to hold the β_2m-HC heterodimer in a peptide-receptive state. β_2m-HC linked chain molecules provide a powerful tool to investigate this theory, since they are synthesised as preformed heterodimer and as a result have no requirement for recruitment of β_2m to heavy chain and might therefore have decreased dependency on calreticulin. For this purpose, the β_2m-B44 construct did not prove useful since it did not reach the cell surface in either K41 or K42 cells. However, the β_2m-A2 construct was able to express in these cells and it was interesting to find that, as with mouse class I molecules this human linked chain molecule still suffered an 8-10 fold reduction in expression in the absence of calreticulin, indicating that low class I expression in calreticulin deficient cells does not result from a defect in the recruitment of β_2m to heavy chain. These findings have formed part of a larger investigation of calreticulin deficiency in class I antigen processing, which has taken place in the rheumatology unit of the Institute of Child Health in London, and has contributed towards a paper, which has been submitted for publication (Fu et al., 2006 under review). The work presented in this chapter has shown that calreticulin deficiency does not lead to a failure in the assembly of β_2m with HC, since preassembled β_2m-A2 heterodimer is still expressed approximately 10-fold less efficiently in calreticulin defcient 42 cells than in 'wild type' K41 cells. Further to this, other work has shown that K42 cells behave somewhat like TAP deficient RMA-S cells (Ljunggren et al., 1990) in that the majority of class I molecules reaching the cell surface are unstable, but can be stabilised by incubation at 26°C. However, in calreticulin deficient cells this phenotype does not result from poor peptide supply since the level of TAP and its efficiency of peptide transport was found

to be unaffected. In fact, the major defect in these cells seems to be an inability to load with peptide when antigen concentration is low. In other words calreticulin is important in lowering the peptide concentration threshold, at which class I molecules will become efficiently loaded. This is shown, by the ability of calreticulin deficient cells to present H2-K^b/SIINFEKL at the cell surface when provided with high levels of full length ovalbumin or ER targeted peptide, but an inability to present antigen provided at lower concentration where presentation by wild type cells is still possible.

There are several possible ways in which calreticulin may enhance the presentation of scarce antigen. Firstly, it may be important in the stabilisation and maintenance of peptide receptive β_2m-HC heterodimer. Although the data presented here suggest that calreticulin is not required for β_2m recruitment to heavy chain, its interaction with the heterodimer may still be important. It may be that calreticulin holds the heterodimer in a conformation that is favourable for peptide binding or that its association with heavy chain stabilises other interactions with the peptide-loading complex and thus indirectly helps in the catalysis of peptide loading. Such a model is attractive, since class I has been shown to traffic at an accelerated rate in K42 cells (Gao et al., 2002), a phenotype that has been shown before to correlate with poor association of class I with the peptide-loading complex (Lewis et al., 1996). However, class I does still associate with the peptide-loading complex in calreticulin deficient cells, suggesting that calreticulin is not crucial for this interaction (Gao et al., 2002). A second possible role for calreticulin lies in its close cooperation with the glucosylation quality control pathway. Calreticulin binds only to N-glucosylated class I molecules. When class I molecules (and other glycopreteins) are correctly folded, the terminal glucose residue of the N-linked glycan, is removed by glucosidase II, disrupting the interaction between class I and calreticulin. This is a generalised pathway for quality control of protein folding in the ER, and like other glycoproteins that are not fully folded, it is possible that peptide-free class I molecules are sensed by UDP-Glucose glycoprotein glucosyltransferase (GT) resulting in the replacement of the terminal glucose residue. Calreticulin binding would then be restored and might therefore promote another round of peptide loading in the peptide-loading complex. The third possible function of calreticulin is that it could promote peptide loading through its association with free peptides. Calreticulin has been shown to bind to peptides *in vitro* (Spee and Neefjes, 1997) and to be able to promote specific immunity against bound peptides (Basu and Srivastava, 1999). Since calreticulin can both bind to peptides and to the developing class I molecule a role in peptide

recruitment could be played by calreticulin, which might allow it to promote the loading of peptides that are at low concentrations in the ER.

It has been previously reported that certain human alleles of MHC class I express poorly in mouse cells because of species differences in tapasin (Peh et al., 1998) and also that even alleles which are supposedly 'tapasin independent' such as HLA-B27 present a quantitavely and qualitatively different repertoire of peptides in the absence of human tapasin (Sesma et al., 2005). Consistent with these problems the expression of β_2m-B44 in murine K41 and K42 cells seems to be impaired. However, expression of human tapasin either by transfection or by transduction with vaccinia virus expressing human tapasin, failed to facilitate cell surface expression of β_2m-B44 in murine K41 cells. β_2m-B44 has been shown to express well in human HCT cells (Fig. 6.3) and in the tapasin deficient human B cell line (Fig. 5.5) demonstrating that the linked chain construct is able to fold normally. K41 cells can therefore be said to suffer from a human tapasin and β_2m independent inability to present certain human MHC class I alleles including HLA-B44.

It has previously been shown that low cell surface class I can be a consequence either of accelerated trafficking of suboptimally loaded MHC class I from the ER to the cell surface or of retention of class I molecules in the ER. The HLA-A2 mutant T134K is a classic example of accelerated trafficking (Lewis et al., 1996). Because of a single amino acid change, this mutant class I allele is unable to interact with tapasin and the peptide-loading complex and as a result exits the ER rapidly after synthesis. However, because they have not been through the normal processing pathway, T134K molecules are not properly loaded with peptide and as a result are unstable at the cell surface and turnover rapidly. Accelerated trafficking to the cell surface therefore leads to a decrease in the steady state level of cell surface class I. This phenomenon is also observed for class I molecules in K42 (calreticulin -/-) cells (Gao et al., 2002), and consistent with this it has been shown here that the reduction in native cell surface class I expression in K42 cells can be recovered by providing the cells with allele specific exogenous class I binding peptide (Fig. 6.7b). However, addition of exogenous HLA-B44 binding peptide failed to enhance expression of β_2m-B44 in K41 cells (Fig. 6.4), indicating that the observed impairment of β_2m-B44 expression in these cells is not a consequence of accelerated trafficking to the cell surface.

Reduced cell surface expression of class I as a result of retention in the ER, seems to occur when class I molecules cannot be properly loaded with peptides. This occurs in Daudi (human β_2m -/-) cells in which a peptide receptive β_2m-HC heterodimer cannot be formed (Williams et al., 1989), and also in TAP deficient cells such as T2 (Paulsson et al., 2001). When class I heterodimer (β_2m-HC) is unable to load with high affinity peptide it is usually retained in the ER, but incubation at lower temperature may allow cell surface expression of these poorly loaded class I molecules. Whereas at 37°C, peptide free or low affinity peptide bound heterodimer would not be stable enough to traffic first to the Golgi body and then on to the cell surface, at 26°C it is more stable and is able to reach the cell surface. The expression of β_2m-B44 is unaffected by incubation at 26°C indicating that it is not forming an unstable peptide-free heterodimer in the ER.

It is interesting to note that with the exception of β_2m deficient cell lines, most reports of poor expression of class I have usually referred to a reduction rather than an absence in cell surface expression of class I. For instance in murine cells even when human β_2m is not provided there is substantial expression of many alleles of human class I (Irwin et al., 1989), and when HLA-B44 is expressed with human β_2m in murine L-cells not expressing human tapasin, there is around a 10-fold increase in surface staining by W6.32 antibody (Peh et al., 1998). It is said that HLA-B44 expresses at the cell surface at 'reduced' levels in this scenario, in that provision of human tapasin will facilitate a higher level of expression. However, this is fundamentally different from the situation shown here, where surface expression of β_2m-B44 in K41 and K42 cells is completely lacking. It is perhaps less surprising then that it is found that co-expression of human tapasin, provision of exogenous peptide and cooling the cells to 26°C all fail to enhance expression of β_2m-B44. Yet the β_2m-B44 construct is able to fold correctly inside K41 and K42 cells, as evidenced by intracellular W6.32 staining and it is also able to express at the cell surface of human cells transduced with the same virus.

The failure of β_2m-B44 to express at the surface of K41 cells despite the fact that it expresses well in human cells implies that there is another component necessary for antigen presentation by HLA-B44, which is present in human HCT cells but not in the mouse cell line K41. A report of a similar phenomenon was made in a recent publication using a mutant cell line 4S8.12 derived from African green monkey COS7 kidney cells (York et al., 2005). In this mutant monkey cell line, transfection with mouse class I alleles allowed cell surface expression of murine class I. Furthermore ovalbumin protein could be processed into suitable peptide, loaded onto Kb and

presented to T cells. However, transfected human and primate class I molecules were unable to traffic to the cell surface. The explanation proposed for this deficit was the failure of β_2m to assemble with heavy chain; however it is interesting that the deficiency was still present when a covalently linked β_2m-HLA-A3 protein was expressed in these cells. This would imply that it is not recruitment of β_2m to heavy chain that is deficient in these cells, but rather that there is a defect at a later stage of processing which prohibits the trafficking of class I. It is interesting that both in the work of York et al. and in the work presented here, the cells are proficient in surface expression of mouse class I molecules but fail to express at least some human class I alleles. The failed surface expression of human class I cannot be rescued in either situation by provision of suitable binding peptide, and there is no evidence that the defect is due to tapasin. Both findings point towards an as yet unidentified component required for folding and subsequent trafficking of at least some alleles of human and primate class I molecules, which is not necessary for mouse class I trafficking.

However, some differences do exist between the results reported here and those of York et al.. Firstly the 4S8.12 cell line if anything exhibits enhanced cell surface expression of mouse MHC class I, even in the presence of primate heavy chains which are unable to appear on the surface. In contrast, expression of the β_2m-B44 construct in K41 causes a downregulation of mouse MHC class I. Secondly the 4S8.12 mutant displays a biphasic phenotype in relation to primate class I expression in which a minority of cells display normal levels of class I; such a population is not seen in K41 cells endogenously expressing β_2m-B44 which unanimously fail to express this construct at the cell surface. It is possible that the two similar phenotypes discussed here have different causes or that other species differences between the monkey COS7 cell and the mouse K41 cell cause the more subtle differences in phenotype. It is also possible that the a greater stability of the linked chain β_2m-B44 causes it to remain in the ER for a long period of time and to occupy proteins of the class I machinery, whereas the monkey or human heavy chain in COS7 cells may be more rapidly degraded if it fails to fold correctly for peptide binding. Despite these differences there is a striking similarity between the phenotype shown by the 4S8.12 mutant and by K41 cells transfected with β_2m-B44.

It is interesting to note that the β_2m-A2 construct expresses at the surface of K41 cells, since it shows that the requirement of this unknown component for surface expression of HLA-B44 is an allele specific phenomenon with relation to human class I molecules. The reduction in mouse class I expression of cells expressing β_2m-B44 as compared to β_2m-A2, implies that when unable fold in such a way as to receive peptide in these cells,

the β_2m-B44 construct impedes mouse class I molecules loading with peptide. Rather than the explanation suggested by Rock et al. (York et al., 2005), that the defect results from a failure of heavy chain and β2m fail to associate, the model proposed here is that the β_2m-HC heterodimer must be chaperoned in such as way that it can bind to available peptide. If this does not occur the molecule will fail to load with peptide and instead will remain associated with other components of class I processing, such as the peptide-loading complex, hence the reduction in mouse class I in K41 cells expressing β_2m-B44 but not in those expressing β_2m-A2.

In conclusion, data presented in this chapter have shown that calreticulin does not play a crucial role in β_2m recruitment to class I heavy chain. This has formed part of a larger project, the data from which points to a role for calreticulin in lowering the peptide concentration threshold at which class I molecules can become efficiently loaded. Other data presented in this chapter suggest, that at least for some human heavy chain alleles, a hitherto unidentified component of class I folding may exist which is required for presentation of class I at the cell's surface.

7 Discussion

MHC class I antigen presentation is central to the cellular arm of the immune system. It is vital for the clearance of many pathogens and may play an important role in the prevention of cancer. Increasing our understanding of MHC class I antigen processing and the production of effective cellular immunity could be instrumental in the development of future treatments for infectious diseases, cancer and autoimmunity.
The work presented in this thesis has focused on the mechanisms that occur in the ER to allow the successful presentation of antigen by MHC class I molecules. This process requires the pairing of class I heavy chain with β_2m, followed by the loading of peptide onto the binding groove of the heavy chain and the export of class I molecules to the cell surface. A number of chaperones are required for this process to take place efficiently and despite, or perhaps because of, the numerous different approaches used to determine the roles of these chaperones, their functions remain unclear.
What is generally accepted is that in normal cells, heavy chain interacts first with calnexin and then later with the peptide-loading complex. Expression of linked chain class I constructs in different cell lines in this work has allowed an examination of these processes and the chaperones involved.
Although clear functions for individual chaperones remain illusive, several lines of evidence suggest that for most alleles the peptide-loading complex is the key player in ensuring that class I molecules are efficiently loaded with peptide when they leave the ER. Knockout cell lines for tapasin (Ortmann et al., 1997), calreticulin (Gao et al., 2002) and most recently ERp57 (Garbi et al., 2006) all show reduced efficiency in peptide loading and in each case at least some alleles of class I dissociate from the peptide-loading complex and traffic to the cell surface at accelerated rate. Similarly the HLA-A2 mutant class I molecule T134K does not interact with tapasin or the peptide-loading complex, traffics quickly to the cell surface and is found to poorly optimise its peptide cargo (Peace-Brewer et al., 1996). Conversely the mutant HLA-A2 heavy chain allele S132C, which is shown to make a prolonged interaction with the peptide-loading complex and becomes endo H resistant more slowly than its wild type counterpart, is found to be very stable at the cell surface and the peptide groove is found to be bound at high occupancy (Lewis et al., 1996). Apart from its role in the provision of peptides the requirement of a functional TAP in the peptide-loading complex is controversial. Some researchers find that surface class I expression can be restored in tapasin deficient cells

by transfection of soluble form of tapasin, which interacts normally with class I molecules but does not interact with TAP (Lehner et al., 1998). This suggests that the crucial interaction for peptide loading is made between class I and tapasin, and that TAP itself is not a required component of the peptide-loading complex. Other researchers have shown that the presence of TAP in the peptide loading-complex is important to class I peptide loading (Tan et al., 2002) and further to this, it has been reported that both nucleotide binding by TAP (Knittler et al., 1999) and the peptide occupancy of TAP (Owen and Pease, 2001)(regardless of actual peptide transport) may be important in the release of class I molecules from the peptide-loading complex. These differences may partly be explained by the observation that the absence of tapasin can cause a decrease in the rate of peptide transport by TAP. This occurs either because TAP is unstable and expressed at lower levels in the absence of tapasin (Garbi et al., 2003) or is impaired in its function by reduced peptide binding efficiency (Li et al., 2000). Since this effect seems to be dependent on cell type (Grandea, III et al., 2000) and since different alleles display different dependencies on tapasin and TAP (Peh et al., 1998) variations in results from different groups may also partly result from differences in the experimental system used.

Regardless of the exact role of each component of the peptide-loading complex the overall function is to facilitate or optimise peptide binding to class I in the ER. Two different simple models can be envisaged that would allow the peptide-loading complex to carry out this function. In the first 'sensing' model, class I molecules are released from the peptide-loading complex only when they have become efficiently bound to high affinity peptide. This model might allow a peptide editing function, but would require a sensing mechanism, which retained empty class I while releasing peptide loaded class I. In the second 'automatic' model, class I molecules progress to the peptide-loading complex by default and are retained there for a period of time defined by their binding affinity for tapasin and other members of the complex. During this period of time, proteins of the peptide-loading complex would provide an environment favourable for binding of available peptides to class I heavy chain. Because class I molecules are not immediately released from the peptide-loading complex there would then be an opportunity for low affinity peptides to dissociate from class I and for another round of peptide binding to occur.

The sensing model is supported by several key findings, most importantly that provision of high affinity peptide leads to allele specific dissociation of class I from the peptide-loading complex (Suh et al., 1994). Conversely, in TAP mutant T2 cells, which cannot

import peptide into the ER from the cytoplasm, the interaction between tapasin and class I is found to be prolonged, resulting in the retention of class I molecules in the ER (Paulsson et al., 2001). The conformation of class I molecules changes upon binding of peptide, as evidenced by specific antibodies able to distinguish between peptide loaded and peptide free class I molecules (Lie et al., 1991). This change in conformation could be sensed by tapasin (Hansen et al., 2005) or other members of the peptide-loading complex, allowing release of peptide loaded class I molecules (Elliott, 1997).

In chapter 5, this sensing model was tested using the PB construct, which consists of an HLA-A2 binding peptide from flu matrix protein linked to β_2m. PB was shown to fold express efficiently at the cell surface with HLA-A2, and was shown to elicit a powerful T cell response from flu specific T cells, showing that the peptide binds efficiently to the groove of the heavy chain. Furthermore, the peptide groove of HLA-A2 was unavailable to bind other peptides at the cell surface as indicated by the failure of HCT PB cells to present another HLA-A2 restricted peptide AAGIGILTV to specific CTL. PB was also shown to be unable to pair up and express with heavy chain molecules other than HLA-A2 when expressed endogenously in Daudi cells. These data suggest that PB pairs with only HLA-A2 in the ER and that the peptide is bound to the binding groove of HLA-A2. Interestingly despite this evidence that the high affinity peptide of PB is bound to the groove of HLA-A2, PB is still found to traffic via the peptide-loading complex as shown by immunoprecipitation with both TAP and tapasin. This finding implies that class I molecules bound to high affinity peptide can be retained by the peptide-loading complex.

Immunoprecipitation of TAP and tapasin from Daudi cells expressing PB would provide stronger evidence that PB is not present in the peptide-loading complex with heavy chain alleles other than HLA-A2. However, since immunoprecipitation requires a large number of cells it would only be practical to do this using a stable cell line expressing PB. Time constraints have not yet allowed for production of this stable cell line. However, other research has also isolated high levels of class I molecules bound to radioactive peptide from tapasin immunoprecipitates in both murine and human cells (Li et al., 1999;Li et al., 2000). Since the vast majority of tapasin is found in association with TAP in normal cells (Diedrich et al., 2001) it seems these tapasin associated, peptide loaded class I molecules have also been retained by the peptide-loading complex. These findings support the automatic model, where class I molecules traffic to the peptide-loading complex by default and dissociate from it in a peptide independent fashion.

Neither simple model is entirely satisfactory, but either can be modified to explain the experimental data. The sensor model could incorporate the idea of retaining loaded class I molecules. If the conformational change in class I upon peptide binding did not trigger immediate release from the peptide-loading complex but merely a lowering in affinity between the peptide-loading complex and class I then this would allow for the recovery of some loaded class I molecules from the peptide-loading complex as shown in this work and by Li et al., and would also allow peptide binding *in vitro* to catalyse the release of class I from TAP. This modified model would also ensure that class I molecules that become loaded with low affinity peptide are not released immediately and might have another opportunity to be reloaded with high affinity peptide.
Alternatively, the automatic model could be modified by adding a further quality control step. Class I molecules would still leave the peptide-loading complex according to a fixed half-life related to their affinity for the peptide-loading complex but poorly loaded class I molecules that dissociated from the peptide-loading complex would be somehow retained in the ER and returned to the peptide-loading complex. This would allow for the retention of class I molecules in TAP deficient cells and explain why the allele specific recovery of class I from the peptide-loading complex gradually falls during incubation with binding peptide. Calreticulin is one candidate, which could fulfil the role of 'retaining and recycling' unloaded class I since in cooperation with UDP Glucosyl transferase it is responsible for the retention of poorly folded glycoproteins. Alternatively the Bap31 (B cell associated protein 31) export system might exclude empty class I molecules from exit sites in the ER causing them to be retained in the ER and return to the peptide-loading complex. Although recycling doesn't seem to occur in the case of the T134K HLA-A2 mutant or in tapasin deficient cell lines in which the majority of class I molecules that reach the cell surface are peptide free, it is possible that since these molecules are not incorporated into the default pathway for class I molecules, they leave the ER via the 'bulk flow pathway' rather than by Bap31 controlled export.
The data presented in this thesis support a model for peptide optimisation in which class I molecules can be retained by the peptide-loading complex even after loading with high affinity peptide. This suggests that the peptide-loading complex does not optimise peptide binding by simply sensing the peptide occupancy of the class I molecule and releasing those molecules that are found to be peptide bound. Instead the mechanism for peptide loading is more subtle, involving automatic retention of peptide-free molecules by the peptide-loading complex and additionally mechanisms for retaining loaded class

I molecules either in the peptide-loading complex or elsewhere in the ER to allow optimisation of their cargo.

Before incorporation into the peptide-loading complex heavy chain is known to interact with calnexin in the 'early complex' (Degen and Williams, 1991). Isolation of this complex using the monoclonal antibody HC10, which recognises heavy chain molecules free from β_2m has indicated the presence of ERp57. In the presence of calnexin but not in its absence, ERp57 is found disulfide bonded to free heavy chain in the early complex (Lindquist et al., 2001;Antoniou et al., 2002), wheras in the peptide-loading complex ERp57 is found disulfide bonded to tapasin and not directly to heavy chain (Dick et al., 2002). An early study of ERp57, reported a disulfide linked intermediate containing ERp57 in the peptide-loading complex (Lindquist et al., 2001) and at the time was assumed to represent heavy chain linked to ERp57. However, in hindsight it seems more likely that the interaction is the same one reported subsequently between ERp57 and tapasin (Dick et al., 2002). The study of interactions between class I heavy chain and both calnexin and ERp57 in the 'early complex' is complicated by the fact that both proteins can also be detected in association with TAP and in some circumstances the peptide-loading complex (Suh et al., 1996;Diedrich et al., 2001;Lindquist et al., 1998). Care must therefore be taken when analysing these data, but the main body of evidence suggests that for human class I alleles, which bear a single N-linked glycan, calnexin is not found in association with heavy chain once it has assembled with β_2m (Zhang et al., 1995;Diedrich et al., 2001). It is surprising then that in the work presented in this thesis, the linked chain molecule β_2m-A2 (BA) was found in association with calnexin. Control experiments showed that free heavy chain also immunoprecipitates with calnexin but that β_2m does not, supporting the accepted model that calnexin, does not normally bind to β_2m-HC heterodimer. The most likely explanation for these apparently contradictory observations is that calnexin is interacting with BA before the protein has been properly folded into its tertiary structure. However, the association of BA with calnexin was strong, so it seems likely that this interaction is persisting beyond the initial stages of protein folding. This could be explained by poor folding of the BA construct in comparison to normal class I. It should be remembered that although the BA linked chain construct has been shown to be functional, it is still an artificial construct and especially given the failure of the PBA construct (peptide-β_2m-A2) to fold, it is possible that the folding of BA is slow, and that it therefore maintains a prolonged interaction with calnexin for the purposes of quality

control. It is also possible that the β_2m of the BA construct somehow disrupts the interaction between HLA-A2 and ERp57. This could impair the formation of disulfide bonds, which are required before the exchange of calnexin for calreticulin can occur. Alternatively, it may be that release of heavy chain from calnexin is independent of β_2m binding. Evidence that β_2m binding causes release of heavy chain from calnexin in human cells is only circumstantial, and the work presented in chapter 5 contradicts such a model. Instead it is possible that both wild type heavy chain and the linked chain construct β_2m-A2 bind calnexin during synthesis. This interaction may persist until initial folding of the heavy chain is complete, when class I molecules could dissociate from calnexin spontaneously. Wild type heavy chain would then associate with β_2m, calreticulin and the peptide-loading complex in the normal way. This model is consistent with the finding that β_2m-A2 seems to bind to calnexin as strongly as wild type heavy chain molecules.

Interestingly despite the numerous reports of interactions between calnexin and class I, no apparent defect in class I presentation has been identified in the absence of calnexin (Scott and Dawson, 1995;Sadasivan et al., 1995;Prasad et al., 1998). Chaperone redundancy has been cited as a possible explanation for this somewhat surprising finding, with the major candidates for replacing the function of calnexin being BiP and possibly calreticulin (Prasad et al., 1998;Balow, 1995). Although such ideas of redundancy have been fashionable in modelling mechanisms of evolution and Darwinian theory (Bruggeman et al., 2000;Miglino and Walker, 2002), other evidence suggests that the presence of proteins with redundant functions are not prolonged during this process of evolution (Ganfornina and Sanchez, 1999), and that duplicated genes, which are not functionally useful either become pseudogenes or 'deteriorate' (are lost from the genome) (Meyer, 1998). Indeed claims of chaperone redundancy between Skp, Sur A and Deg O of E. coli (Rizzitello et al., 2001) were later proved to be incorrect with specific functions later assigned to both Sur A (Justice et al., 2005) and Deg P (Castillo-Keller et al., 2006). In the latter case it was found that under normal conditions Deg P deficient cells appeared normal, but that when a mutant outer membrane protein was expressed it was lethal in the absence of Deg P. Although it is possible that calnexin is redundant for class I assembly it is more likely that it plays a role, which we do not fully understand. Calnexin, like Deg P, may not be important for class I molecules that fold correctly, but may be involved in the degradation of those that don't. It has been shown that calnexin is necessary for the recruitment of ERp57 to free heavy chain (Lindquist et al., 2001) and independently that ERp57 is important in

disulfide bond reduction of class I molecules that fail to fold properly (Antoniou et al., 2002). Calnexin along with ERp57 might therefore play an important role in the unfolding of class I molecules that fail to mature, allowing them to be translocated into the cytoplasm for degradation (Hughes et al., 1997).

Further to its involvement in the 'early complex', ERp57 is also found in the peptide-loading complex (Lindquist et al., 1998;Dick et al., 2002). Data presented in chapter 3 described attempts to inhibit the expression of ERp57 using RNA interference, with a view to exploring the various functions carried out by ERp57, first in the early complex, and then in the peptide-loading complex. Evidence that ERp57 is disulfide bonded to tapasin in the peptide-loading complex suggested that the two proteins might be cooperating in a system for peptide binding or optimisation (Dick et al., 2002). ERp57 contains 2 thioredoxin domains each containing an active CXXC motif, capable of catalysing disulfide bond isomerisation (Hirano et al., 1995;Urade et al., 1997). ERp57 is bonded to tapasin via Cys-57 of the ^{57}CXXC^{60} motif and mutation of Cys-60 to alanine traps this intermediate. The site of binding on tapasin is Cys-95 and mutation of this residue leads to loss of ERp57 from the peptide-loading complex (Dick et al., 2002). Despite the loss of interaction with ERp57, the Cys-95 mutant tapasin was able to restore cell surface expression of HLA-B44 in tapasin deficient cells and the interactions of HLA-B44 with tapasin and the pepide-loading complex were unaffected implying that ERp57 recruitment is not required for HLA-B44 expression. However, the phenotype was not entirely normal in these cells, since HLA-B44 displayed increased turnover at the cell surface suggesting a failure of efficient peptide loading. This inefficiency in peptide loading was confirmed by a radioactive peptide binding assay. These data suggest that the major functions of ERp57 in peptide binding and the folding of normal class I molecules occur in the peptide-loading complex, and this is supported by the fact that failure of ERp57 to associate with immature class I molecules in calnexin deficient cells does not affect class I expression. It was hypothesised that suppression of ERp57 might lead to a class I phenotype similar to that observed with the Cys-95 tapasin mutant, confirming the role of ERp57 in the peptide-loading complex. Unfortunately it was not possible to suppress ERp57 sufficiently to observe any change in surface class I expression using RNA interference, either in the work presented in chapter 3 or in attempts made by Cresswell et al. (personal communication) or Williams et al. (Williams, 2006). However, in 2006 data have been published using a mouse knockout for ERp57 (Garbi et al., 2006). The phenotype is found to be similar to

tapasin deficient cells expressing Cys-95 mutant tapasin, in that class I turnover is increased at the cell surface. Both overall expression and cell surface expression of H-2K^b is reduced about 2-fold in ERp57 knockout cells but expression of H-2D^b is only slightly affected. In contrast to work with the Cys-95 tapasin mutant, the interaction between heavy chain and the peptide-loading complex in ERp57 deficient cells was found to be 10-fold reduced and the interaction was short lived. Furthermore, H-2K^b molecules exited the ER at an accelerated rate and could be stabilised at the cell surface by addition of H-2K^b binding peptide. These findings confirm that ERp57 like tapasin is important in the retention of some class I alleles in the ER for proper peptide loading. It seems that the dependence of different class I molecules on ERp57 does not necessarily follow the same pattern as tapasin dependence, since whereas both H-2D^b and H-2K^b are thought to be dependent on tapasin only H-2K^b expression was significantly altered by lack of tapasin. This finding is somewhat surprising given that H-2D^b was found to associate weakly with tapasin in ERp57 deficient cells but apparently displayed normally at the cell surface. It is possible that dependence on ERp57 is governed by the requirement of class I molecules for disulfide bond formation in the peptide-loading complex. Interestingly, it has been observed that in Cys-95 tapasin mutant cells, which fail to recruit ERp57 to the peptide-loading complex, HLA-B44 is incompletely oxidised in the peptide-loading complex. Cells expressing normal tapasin contain only fully oxidised class I molecules in the peptide-loading complex (Dick et al., 2002). ERp57 may therefore play a key role in maintaining or forming the disulfide bond of the α2 domain of heavy chain, which is likely to be exposed in the absence of peptide. The persistence of the interaction of ERp57 and tapasin is unusual for members of the protein disulfide isomerase (PDI) family, since usually the function of the PDI is to catalyse folding and then dissociate from its substrate (Huppa and Ploegh, 1998). The persistence of this interaction ensures that ERp57 is always present in the peptide-loading complex and available to catalyse disulfide bond isomerisation of the heavy chain. When bound to tapasin via the ^{57}CXXC^{60} motif the free ^{406}CXXC^{409} motif of ERp57 is found in both reduced and oxidised states, confirming that it is available to undergo redox reactions in the peptide-loading complex and supporting the hypothesis that its role in class I folding involves the catalysis of disulfide bond isomerisation (Antoniou and Powis, 2003).

The various roles for tapasin, calreticulin and ERp57 in peptide loading, peptide optimisation and retention of unloaded class I molecules are still not fully elucidated.

Assigning particular roles to each molecule is difficult, since each component is important in maintaining the stability of the peptide-loading complex and as a result knockout cell lines for each of the molecules show overlapping functions. But I will now attempt to summarise the current understanding of these molecules from the body of literature and the evidence provided in this thesis.

The possible roles of ERp57 in the complex were discussed to some extent earlier and a role in the isomerisation of the disulfide bond of the α2 domain of the heavy chain is an attractive one since ERp57 is an oxidoreductase enzyme. However, the conundrum here remains that heavy chain molecules isolated from the peptide-loading complex of ERp57 deficient cells are reported to be fully oxidised just as they are in wild type cells (Garbi et al., 2006). In contrast, Dick et al. reported that HLA-B*4402 molecules recovered from peptide-loading complexes lacking ERp57 were partly reduced (Dick et al., 2002). It is possible that the requirement for ERp57 to fully oxidise class I molecules is an allele specific phenomenon and future work using a recently created ERp57 knockout cell line (Garbi et al., 2006) may help to clarify this. One likely approach for this would be to transfect into ERp57 knockout cells, mutant forms of ERp57, which retain their ability to bind tapasin through Cys-57 but are mutated at the C-terminal CXXC motif and are therefore unable to catalyse other redox reactions. This would allow separate assessment of the importance of ERp57 in stabilising the peptide-loading complex and in oxidation of class I.

Tapasin is the most likely candidate for a peptide editing function – if indeed one exists at all. Unlike calreticulin and ERp57, tapasin is thought to be a dedicated molecule for class I processing (Diedrich et al., 2001) and as such may be more likely to be able to carry out a specialised function such as peptide editing. Moreover, tapasin is thought to bind to residues of the heavy chain in or surrounding the peptide groove (Yu et al., 1999), giving it access to the crucial residues that are likely to shift upon peptide binding. Recently a study of peptide binding to class I has also implicated a role for tapasin in peptide selection (Howarth et al., 2004). In this study a range of similar peptides which, when bound to H2-K^b variably stabilise its expression at the cell surface, were assayed for peptide loading efficiency in several cell lines. In wild type cells the peptide SIINFEKL, which most efficiently stabilised H2-K^b expression was also found to be loaded most efficiently onto class I molecules in the ER; binding of the other peptides showed a similar pattern with a correlation between stability of the K^b-peptide complex at the cell surface and the efficiency of peptide loading. However, in tapasin deficient cells this hierarchy was lost so that the most stabilising peptide

SIINFEKL was now bound to K^b at less than half the rate than the peptide SIINFEKM, which forms a relatively unstable complex with K^b. This experiment is unable to distinguish fully between the function of tapasin and that of the peptide-loading complex as a whole since the stability of the whole peptide-loading complex is compromised by the absence of tapasin. However, in calreticulin deficient cells or cells in which ERp57 is not recruited to the peptide-loading complex the hierarchy of peptide binding remains the same as in wild type cells. Tapasin therefore seems to specifically enhance the presentation of peptides that bind to class I with high affinity in preference to those with lower affinity. Peptide elution assays from class I molecules of normal and tapasin deficient cells have been able to confirm that the class I peptide repertoire of tapasin deficient cells is significantly different from that of wild-type cells, however the peptides eluted in the absence of tapasin were not found to be of lower affinity than in wild type cells (Zarling et al., 2003). However, elution assays take only a snapshot of the peptides bound at the cell surface and do not necessarily represent the distribution of peptides leaving the ER, since low affinity peptides would be turned over quickly at the cell surface and would be recovered at low frequency by elution.

The function of calreticulin in MHC class I antigen processing is an area of active research in the group of Dr. Gao with whom the work presented in this thesis has been carried out. Work from this thesis and from others in the group has led to a greater understanding of the role of calreticulin in peptide loading and class I expression. It has previously been shown that class I molecules traffic at an accelerated rate in calreticulin deficient cells (Gao et al., 2002); further to this it has now been possible to show that this accelerated trafficking leads to unstable expression of class I at the cell surface, which can be restored by addition of exogenous peptide (Fig. 6.7) or by incubating the cells at 26°C. This phenotype is somewhat analogous to that observed in TAP deficient RMA-S cells, which also express unstable class I molecules at the cell surface. However, expression of tapasin and TAP by western blot and the function of TAP assessed using a peptide transport assay, are found to be unaffected in calreticulin deficient cells implying that calreticulin has a direct role in peptide loading. The fact that the peptide-loading complex forms and incorporates class I molecules in calreticulin cell lines along with reduced surface expression of class I supports this. Binding of heavy chain to β_2m and exchange of calnexin for calreticulin are thought to occur simultaneously (Sadasivan et al., 1996) suggesting that calreticulin might be important in the recruitment of β_2m to the heavy chain. This theory was tested in chapter 6 by expressing the linked chain molecule β_2m-A2 in both calreticulin deficient

(K42) and wild type (K41) cells, since β_2m is already covalently linked to HLA-A2 upon synthesis of the β_2m-A2 construct. However, it was shown that the phenotype of β_2m-A2 in calreticulin deficient cells was similar to that of wild type class I, displaying an 8-10 fold decrease in cell surface expression (Fig. 6.2). The deficiency in surface class I expression in calreticulin deficient cells does not therefore result from a failure to recruit β_2m to heavy chain. Instead data produced by H. Fu indicate that in calreticulin cells the defect is in the ability of cells to present scarce peptide at the cell surface. In order to elicit a detectable response from a B3Z T cell hybridoma specific for the ovalbumin derived peptide SIINFEKL, calreticulin deficient cells had to be pulsed with 4-fold higher concentration of ovalbumin than wild type cells. From these results, it is hypothesised that calreticulin lowers the concentration threshold for antigen to be loaded onto class I molecules and expressed at the cell surface. It is not clear how calreticulin facilitates the binding of scarce antigen to class I. However, possible mechanisms include: stabilisation of the β_2m-HC heterodimer and/or the peptide-loading complex, cooperation with the UDP glucose system of quality control for ER proteins, and enrichment of peptides through the ability of calreticulin to bind to peptides and deliver them to the peptide-loading complex. These possible mechanisms were discussed extensively in section 6.4.

Evidence from the work of York et al. (York et al., 2005) and from chapter 6 suggests that some human alleles require another unidentified component in order to be properly processed. This component is not present in mouse fibroblast K41 cells or a mutant African Green Monkey COS7 cell line 4S8.12. HLA-A2 and mouse class I molecules do not seem to have any requirement for this component, conversely HLA-B44 and HLA-A3 cannot be presented in its absence. Data shown in chapter 6 confirmed that human tapasin could not recover cell surface expression of the linked chain molecule β_2m-B44 in K41 cells showing that the required component was not a protein previously identified as being necessary for human class I processing in murine cells. Conversely, it was shown that β_2m-B44 expresses normally in human HCT cells and that the similar linked chain molecule β_2m-A2 is able to express in K41 cells. The expression of linked chain β_2m-B44 in K41 cells could not be recovered by addition of exogenous HLA-B44 binding peptide. Class I molecules that traffic rapidly to the cell surface but are then quickly degraded are stabilised by adding exogenous peptide (Peh et al., 1998), so β_2m-B44 is presumably not trafficking to the cell surface at all in K41 cells. The defect in β_2m-B44 presentation does not therefore arise from a failure to be

efficiently recruited to the peptide-loading complex as seen in cells deficient in tapasin (Peh et al., 1998) or ERp57 (Garbi et al., 2006). These other deficiencies also allow some cell surface expression of class I although at a lower level than in wild type cells, in contrast the absence of this novel component leads to a complete failure of class I expression at the cell surface. The stage at which β_2m-B44 is being retained in the ER in K41 cells is not clear but it is interesting that in COS7 cells, which display a similar phenotype, York et al. find that the formation of β_2m-HC heterodimer is impaired (York et al., 2005), suggesting that the defect occurs early in the process of class I folding. However, it is clear that it is not a deficiency in the recruitment of β_2m that is inhibiting expression of β_2m-B44 in K41 cells since the HLA-B44 is already linked to β_2m in this construct. Interestingly the expression of native mouse class I is reduced in K41 cells when β_2m-B44 is introduced. This is perhaps not surprising since β_2m-B44 is being expressed in the ER but is not progressing from it, and is therefore likely to be interacting with proteins which are usually involved in mouse class I folding. Inhibition of mouse class I folding by β_2m-B44 at an early stage of assembly is consistent with other findings of competition between class I alleles at this stage of development (Tourdot et al., 2005). It is possible that expression of HLA-B44 might inhibit mouse class I even if it were processed normally, but it seems likely that the failure of β_2m-B44 to leave the ER is exacerbating this effect, especially given the fact that the β_2m-A2 construct, which is expressed normally at the cell surface does not alter mouse class I expression. It is not clear whether the 'missing' component for β_2m-B44 expression is absent in K41 cells or whether the murine protein expressed is unable to carry out the function that is performed by its human counterpart. It is possible that β_2m-B44 competes with mouse class I molecules for this component and since β_2m-B44 cannot be correctly folded, mouse class I expression is inhibited by lack of access to this component. However, expression of HLA-B44 and other human alleles has been demonstrated successfully in other murine cells (Peh et al., 1998), so it is not clear why there would be a species compatibility issue for this component in K41 cells. It is possible that K41 cells express a mutated or unusual allele of this protein, which does not facilitate folding of HLA-B44 or that it is absent in these cells. Mouse class I molecules do not seem to have an absolute requirement for this component since expression of mouse class I is normal both in K41 cells and in the COS7 4S8.12 mutant cell line (York et al., 2005). It is formally possible that the nature of the β_2m-B44 construct is inhibiting its expression in these cells, however its successful expression in human cells and the successful expression of the similar β_2m-A2 construct in K41 cells

argues against this. It is therefore hypothesised that a hitherto unknown component is required for cell surface expression of HLA-B44 and some other primate alleles. This component is likely to be involved in the early processes of class I folding and cannot be compensated for by expression of human tapasin or provision of peptide.

From the body of information in the literature and the findings discussed here the following pathway is proposed for the processing of class I alleles such as HLA-B*4402 that are dependent early after synthesis for an unknown component of class I processing and then subsequently are also dependent on tapasin and ERp57 (Peh et al., 1998). During and shortly after synthesis, heavy chain is bound to calnexin and is folded with the aid of disulfide bond formation in the α3 domain by ERp57 (Farmery et al., 2000). Subsequently, heavy chain dissociates from calnexin (human alleles) and binds to β_2m and calreticulin (Sadasivan et al., 1996). A hitherto unknown component also assists in the development of class I at this early stage (York et al., 2005)(data presented here); different class I alleles show different requirements for this component and may also compete for it (Tourdot et al., 2005). By default class I molecules are then incorporated into the peptide-loading complex (data presented here). Meanwhile, TAP is transporting peptides into the ER from the cytoplasm (Spies et al., 1992) and ERAAP (Endoplamic Reticulum Aminopeptidase associated with Antigen Processing) trims amino acids one at a time from the N-terminus of imported peptides to produces sequences of 8-9 residues (Serwold et al., 2002;York et al., 2002).
Class I molecules are then held by the peptide loading complex in a state where they are able to bind peptides efficiently. If necessary ERp57 oxidises and then maintains the disulfide bond of the α2 domain of heavy chain (Dick et al., 2002), tapasin monitors peptide binding and optimises the cargo (Howarth et al., 2004), and calreticulin enhances the binding of scarce peptides to the groove of class I (work presented here and H Fu personal communication). Once bound by peptide, class I molecules may have lower affinity for the peptide-loading complex (Suh et al., 1994) but can remain associated with it (data presented here). The half-life of the continuing interaction is controlled by the off-rate of the loaded class I molecule or by deglucosylation of class I by glucosidase II enzyme (van Leeuwen and Kearse, 1996) or by recruitment of loaded class I molecules to ER exit sites by Bap29/31 (Paquet et al., 2004). Class I molecules that lose their peptide after dissociation from the peptide-loading complex may be recycled to the peptide-loading complex to optimise peptide loading (Paulsson et al., 2002). Otherwise, peptide loaded class I molecules are transported out of the

endoplasmic reticulum in coat protein complex II (COPII) vesicles and traffic via the Golgi body to the cell surface.

An understanding of MHC class I presentation is important for medical science, since stimulating or controlling cellular immunity is likely to provide powerful tools for fighting infectious disease and cancer, generating new vaccines, and controlling autoimmunity. In order for an effective CD8+ T cell response to be mounted several crucial encounters must occur between the T cell receptor (TCR) and MHC class I molecules. If any of these encounters is disrupted then cytotoxic T cell immunity will fail and the offending cells will not be destroyed.

In the thymus T cells are both positively and negatively selected according to the interaction between the TCR and self-antigens presented by MHC class I. In the absence of MHC class I expression of cells in the thymus, CD8+ T cells do not develop (Huesmann et al., 1991); conversely the interaction between the TCR and the MHC/peptide complex must not be too strong as this leads to negative selection of the T cell. This negative selection eliminates T cells whose TCR has a high affinity for self-antigens and is thought to be an important defense mechanism against autoimmunity (Kappler et al., 1987). Naive CD8+ T cells, which have successfully undergone this selection process leave the thymus and enter the circulation. In order to become activated and produce effector cells, T cells must encounter specific antigen presented in the correct context. This is thought to involve presentation of specific antigen usually by dendritic cells (DCs) (Rock et al., 1993) (Irwin et al., 1989) in conjunction with danger signals such as TLRs (Toll-Like Receptors) and cytokines. Once activated, T cells circulate in the periphery and upon recognition of specific antigen at the surface of target cells they can finally undertake killing.

Until recently, most work on the interactions between class I and the TCR focused on presentation of antigen by the target cell. This process requires the classical pathway of class I antigen presentation, whereby intracellular proteins are broken down by the proteasome and antigens derived from these proteins are transported into the ER by TAP and loaded onto developing class I molecules (Fig. 1.1). This process probably occurs in the majority of nucleated cells in the body and is sensitive to brefeldin A, which blocks transport through the Golgi body, and to lactacystin and other proteasome inhibitors. However, this model, in which class I is responsible only for presentation of intracellular antigens, cannot adequately explain the efficacy of many vaccines or the

development of immunity against pathogens, which do not infect macrophages or dendritic cells. Development of immunity in these situations necessitates the processing of extracellular antigens on class I molecules, a phenomenon termed 'cross presentation' (Bevan, 1976). Some research has suggested that cross presentation by dendritic cells and other cells might simply involve the release of phagocytosed proteins into the cytosol followed by presentation via the classical pathway for presentation of intracellular antigens (Kovacsovics-Bankowski and Rock, 1995). But other research has shown that some antigens can be cross-presented on class I molecules in the presence of the Golgi blocker brefeldin A, suggesting a separate pathway for presentation, which does not involve transport through the Golgi (Pfeifer et al., 1993). It was suggested that an alternative pathway might exist where class I molecules enter the normal pathway of class II molecules and become loaded with antigens in the phago-lysosomal pathway, allowing cross presentation of exogenous antigens on class I.

Recent work has to some extent combined some of the ideas of these two pathways. During phagocytosis in macrophages there was shown to be a fusion between membrane of the ER and the developing phagosome (Gagnon et al., 2002). A similar process was demonstrated in dendritic cells where the phagosome formed was shown to contain the ER proteins known to be necessary for antigen processing such as TAP, tapasin, calreticulin, ERp57 and ERAAP but not general ER proteins such as (Ackerman et al., 2003). Furthermore, the presence of Sec61 on and the close association of proteasomes with phagosomes indicated that there was an efficient system for retrotranslocation and degradation of phagocytosed proteins (Houde et al., 2003). It may be that these phagosomes therefore constitute a specialised compartment to allow the highly efficient presentation of exogenous antigens, which has been demonstrated by dendritic cells (York et al., 2005;Kovacsovics-Bankowski et al., 1993). In this model loading would occur in phagosomes but still requires the normal machinery for class I presentation including the proteasome and TAP. Although proteins exported from the phagosome and broken down by the proteasome could in theory then enter the ER pathway for antigen presentation, it seems that the majority of class I becomes loaded with peptides that have returned to the phagosome (Ackerman et al., 2003). Another separate pathway for cross presentation, which is independent of TAP and the proteasome, but dependent on cathepsin S is still thought to exist in macrophages but its contribution to immunity *in vivo* remains unclear (Huang et al., 1996;Shen et al., 2004). An understanding of the events of class I presentation at this

priming stage is crucial to the design of vaccines, which aim to exploit the presentation of antigens by dendritic cells to produce a strong CD8+ T cell response.
Many viruses and tumour cells have developed strategies to avoid detection and the diversity and widespread use of these mechanisms give us an insight into the importance of class I antigen presentation. Class I presentation is subverted by different viruses at almost every stage, regulation of transcription of antigen presentation genes is altered (Tortorella et al., 2000), class I molecules are degraded by being rerouted from the ER to the lysosome by HIV protein Nef 1(Kasper et al., 2005) or from the ER to the cytosol by HCMV proteins US2 and US11 (Wiertz et al., 1996). Endocytosis of class I from the cell surface is increased by K3 and K5 in sarcoma-associated herpes virus (Coscoy and Ganem, 2003), the import of peptides by TAP is blocked by HSV-1 protein ICP47 (Neumann et al., 1997) and HCMV protein US6 (Hewitt et al., 2001), while HCMV protein US3 interacts with and may interfere with the function of tapasin (Park et al., 2004). The fact that the highly compact genome of viruses contains these class I evasion mechanisms shows how important avoidance of CTL detection has been to their survival, and it is important to take these mechanisms into account when thinking about vaccination. After all, even if a vaccine successfully primes T cells, the T cell response will not be effective unless target cells also display the specific antigen recognised by effector T cells.

In the future it would be interesting to study the ability of PB constructs similar to that used in this work, in which peptide is attached to β_2m, to stimulate T cell responses. The transfection of HCT cells with PB restored surface class I to normal levels and since all the class I molecules at the surface of these cells are presenting the same peptide this could provide powerful stimulation for T cells. *In vitro* it would be possible to introduce the PB construct into dendritic cells and assess their ability to stimulate naïve T cells. During this project, high titre virus expressing the linked chain construct PB and also a virus able to coexpress PB and the TAP inhibitor ICP47 (data not shown) have been produced and these viruses could be used in for this purpose. Coexpression of PB and ICP47 would be an interesting tool for the stimulation of CD8+ T cells. In culture it has been possible to expand T cell clones to great efficiency (100-fold expansion / 2 week stimulation – data not shown) using the anti-CD3 antibody OKT-3 and carefully controlled culture conditions. *In vitro* stimulation of patient T cells, with known antigens derived from overexpressed proteins has been trialled for the treatment of cancers such as melanoma (Yee et al., 2002). One of the limiting factors in this process

is the generation of sufficient numbers of good antigen presenting cells (usually dendritic cells) to stimulate extracted T cells *in vitro*. If a melanoma specific peptide was linked to β_2m in a fusion protein in a parallel way to the PB construct used in this work, then it could be introduced into antigen presenting cells and would not require TAP to express at the cell surface. Coexpression with ICP47 would block the presentation of other peptides by inhibiting the function of TAP. This could be beneficial in two ways. Firstly, it might increase the amount of specific peptide presented at the cell surface, since the introduced PB construct would not be competing with other peptides for heavy chain, and secondly it might allow the use of allogeneic dendritic cells as antigen presenting cells. These dendritic cells would need to be matched for the heavy chain allele required to bind to the specific peptide used but could be easily obtained in large quantities from normal blood banks. A strong mixed lymphocyte reaction between the patient CD8+ T cells and the allogeneic antigen presenting cells might be avoided, since the only class I expressed by antigen presenting cells would be in association with the PB construct.

Expression of PB in tapasin deficient cell lines or in combination with the HLA-A2 mutant allele T134K would also be interesting as it would allow an analysis of the importance of interactions of class I molecules with the peptide-loading complex independent of the process of peptide loading. This is an interesting question since it has been reported that regardless of their peptide status at the cell surface, class I molecules which have been processed in the presence of tapasin are more stable than those that are processed in the absence of tapasin or in the presence of soluble tapasin (Zarling et al., 2003).

Preliminary DNA vaccinations using the PB construct have been carried out in HLA-A2 transgenic mice to determine its ability to generate effective immunity *in vivo*. The results from the first set of mice indicated an increase in the number of flu-specific T cells in immunised mice but the results are so far inconclusive (data not shown). This work is now being continued by collaborators in The Institute of Microbiology of the Chinese Academy of Science in Beijing.
As discussed earlier, generation of a stable Daudi cell line expressing PB would be useful for purposes of comparison with HCT PB cells. Since the genotype of Daudi cells does not include HLA-A2, immunoprecipitation of TAP and tapasin in Daudi cells

expressing PB would provide direct evidence showing whether PB can associate with other heavy chain alleles in the peptide-loading complex.

The stable cell lines generated in this work could also be used in further experiments. There are a number of further experiments that could be carried out using HCT cells expressing different class I fusion proteins. Since β_2m-A2 seems to be expressed at such a high level it would be interesting to measure its half-life at the cell surface. This can be done by blocking Golgi transport using a reagent such as brefeldin A, and monitoring the loss of surface class I over time by flow cytometry. A similar experiment could be performed for cells expressing the PB construct. Together this would allow an assessment of the contributions of stably bound peptide and of β_2m association in the rate that heavy chain class I molecules are turned over from the cell surface.

Transduction of murine K41 cells with the linked chain molecule β_2m-B44, has shown that K41 cells are deficient in a component required for presentation of HLA-B44. It would be of interest to explore this defect further. By immunoprecipitation it would be possible to determine whether or not β_2m-B44 reaches the peptide-loading complex. A library approach could also be used to introduce human proteins into K41 cells to try and identify the component needed for processing of HLA-B44 and other primate alleles. Although it is unclear which proteins might fulfil this role possible candidates might include Bap29/31 and ERp57, which are both known to interact with HLA-B44. It would also be interesting to further characterise the loss of murine class I from K41 cells endogenously expressing β_2m-B44. For instance, does the loss of murine class I lead to a failure in antigen presentation, and are all mouse class I alleles affected equally by the expression of β_2m-B44 in K41 cells. In order to explore further the requirements for expression of β_2m-B44 it would be interesting to introduce it into other murine cell lines. Separate expression of human β_2m and HLA-B44 should also be investigated in K41 cells to confirm that failure to express it is not a merely a property of the linked chain β_2m-B44 molecule, but also extends to wild type class I. To this end K41 cells expressing human β_2m have already been generated using virus produced in chapter 4 (data not shown).

In summary, the work presented in this thesis has contributed to the understanding of the processes involved in class I antigen processing in the ER and has addressed several key stages in the regulation of class I folding. Work in the murine K41 cell line has demonstrated that HLA-B*4402 has a requirement for hitherto unknown component of

class I antigen processing. In cooperation with other researchers in the group the role of calreticulin has been better characterised. Data presented here were able to show that peptide could stabilise class I molecules at the cell surface of calreticulin deficient cells and excluded failure in β_2m recruitment as the reason for decreased expression of class I in these cells. Instead it was shown (by H. Fu) that calreticulin enriches or somehow facilitates binding of scarce peptides to class I molecules.

Using linked chain molecules it was shown that class I molecules follow a default pathway during their synthesis involving interactions with both calnexin and the peptide-loading complex. β_2m-A2 was shown to bind to calnexin, which is surprising since for human alleles β_2m associated heavy chain molecules are not normally found bound to calnexin (Sadasivan et al., 1996). Linked chain β_2m-HC heterodimers were also shown to bind to the peptide-loading complex and to be dependent on both TAP and tapasin for optimal class I expression.

It has also been possible to show that class I fusion constructs consisting of peptide linked to β_2m (PB) and β_2m linked to HLA-A2 (BA) can express normally in human cells. They are fully functional and can be used to stimulate powerful T cell responses *in vitro*. Such constructs may be powerful tools in the future for use in vaccination as they may allow presentation of specific peptides at high density at the cell surface. Linked chain constructs may also prove useful in tetramer technology where separate expression and refolding of the three components of the mature class I molecule is both laborious and costly.

Most interestingly, the construct consisting of peptide linked to β_2m (PB) was recovered from the peptide-loading complex. Having excluded the possibility that PB is paired with other heavy chain alleles and shown that the peptide groove of HLA-A2 is bound to the peptide of the PB construct and is not available for peptide binding, it is hypothesised from these data that class I molecules bound to high affinity peptide can be retained by the peptide-loading complex. This supports an automatic pathway of class I folding, where the peptide-loading complex acts as a passive catalyst for loading of peptide cargo.

References

1. Ackerman, A.L., C.Kyritsis, R.Tampe, and P.Cresswell. 2003. Early phagosomes in dendritic cells form a cellular compartment sufficient for cross presentation of exogenous antigens. *Proc. Natl. Acad. Sci. U. S. A* 100:12889-12894.

2. Ajitkumar, P., S.S.Geier, K.V.Kesari, F.Borriello, M.Nakagawa, J.A.Bluestone, M.A.Saper, D.C.Wiley, and S.G.Nathenson. 1988. Evidence that multiple residues on both the alpha-helices of the class I MHC molecule are simultaneously recognized by the T cell receptor. *Cell* 54:47-56.

3. Alexander, J., J.A.Payne, R.Murray, J.A.Frelinger, and P.Cresswell. 1989. Differential transport requirements of HLA and H-2 class I glycoproteins. *Immunogenetics* 29:380-388.

4. Anderson, K., P.Cresswell, M.Gammon, J.Hermes, A.Williamson, and H.Zweerink. 1991. Endogenously synthesized peptide with an endoplasmic reticulum signal sequence sensitizes antigen processing mutant cells to class I-restricted cell-mediated lysis. *J Exp. Med.* 174:489-492.

5. Anderson, K.V. 2000. Toll signaling pathways in the innate immune response. *Curr. Opin. Immunol.* 12:13-19.

6. Androlewicz, M.J., K.S.Anderson, and P.Cresswell. 1993. Evidence that transporters associated with antigen processing translocate a major histocompatibility complex class I-binding peptide into the endoplasmic reticulum in an ATP-dependent manner. *Proc. Natl. Acad. Sci. U. S. A* 90:9130-9134.

7. Androlewicz, M.J., B.Ortmann, P.M.van Endert, T.Spies, and P.Cresswell. 1994. Characteristics of peptide and major histocompatibility complex class I/beta 2-microglobulin binding to the transporters associated with antigen processing (TAP1 and TAP2). *Proc. Natl. Acad. Sci. U. S. A* 91:12716-12720.

8. Antoniou, A.N., S.Ford, M.Alphey, A.Osborne, T.Elliott, and S.J.Powis. 2002. The oxidoreductase ERp57 efficiently reduces partially folded in preference to fully folded MHC class I molecules. *EMBO J.* 21:2655-2663.

9. Antoniou, A.N. and S.J.Powis. 2003. Characterization of the ERp57-Tapasin complex by rapid cellular acidification and thiol modification. *Antioxid. Redox. Signal.* 5:375-379.

10. Asano, M., M.Toda, N.Sakaguchi, and S.Sakaguchi. 1996. Autoimmune disease as a consequence of developmental abnormality of a T cell subpopulation. *J. Exp. Med.* 184:387-396.

11. Baas, E.J., H.M.van Santen, M.J.Kleijmeer, H.J.Geuze, P.J.Peters, and H.L.Ploegh. 1992. Peptide-induced stabilization and intracellular localization of empty HLA class I complexes. *J. Exp. Med.* 176:147-156.

12. Balow, J.P., J.D. Weissman, K.P. Kearse. 1995. Unique expression of major histocompatibility complex class I proteins in the absence of glucose trimming and calnexin association. *J Biol. Chem.* 270:29025-29029.

13. Basu, S. and P.K.Srivastava. 1999. Calreticulin, a peptide-binding chaperone of the endoplasmic reticulum, elicits tumor- and peptide-specific immunity. *J Exp. Med* 189:797-802.

14. Berman, S., H.Gewurz, and C.Mold. 1986. Binding of C-reactive protein to nucleated cells leads to complement activation without cytolysis. *J. Immunol.* 136:1354-1359.

15. Bernstein, E., A.A.Caudy, S.M.Hammond, and G.J.Hannon. 2001. Role for a bidentate ribonuclease in the initiation step of RNA interference. *Nature* 409:363-366.

16. Berton, M.T., J.W.Uhr, and E.S.Vitetta. 1989. Synthesis of germ-line gamma 1 immunoglobulin heavy-chain transcripts in resting B cells: induction by interleukin 4 and inhibition by interferon gamma. *Proc. Natl. Acad. Sci. U. S. A* 86:2829-2833.

17. Beutler, B. 2004. Innate immunity: an overview. *Mol. Immunol.* 40:845-859.

18. Beutler, B., Z.Jiang, P.Georgel, K.Crozat, B.Croker, S.Rutschmann, X.Du, and K.Hoebe. 2006. GENETIC ANALYSIS OF HOST RESISTANCE: Toll-Like Receptor Signaling and Immunity at Large. *Annu. Rev. Immunol.* 24:353-389.

19. Bevan, M.J. 1976. Minor H antigens introduced on H-2 different stimulating cells cross-react at the cytotoxic T cell level during in vivo priming. *J. Immunol.* 117:2233-2238.

20. Bicknell, D.C., A.Rowan, and W.F.Bodmer. 1994. Beta 2-microglobulin gene mutations: a study of established colorectal cell lines and fresh tumors. *Proc. Natl. Acad. Sci. U. S. A* 91:4751-4755.

21. Bjorkman, P.J., M.A.Saper, B.Samraoui, W.S.Bennett, J.L.Strominger, and D.C.Wiley. 1987a. Structure of the human class I histocompatibility antigen, HLA-A2. *Nature* 329:506-512.

22. Bjorkman, P.J., M.A.Saper, B.Samraoui, W.S.Bennett, J.L.Strominger, and D.C.Wiley. 1987b. The foreign antigen binding site and T cell recognition regions of class I histocompatibility antigens. *Nature* 329:512-518.

23. Boshart, M., F.Weber, G.Jahn, K.Dorsch-Hasler, B.Fleckenstein, and W.Schaffner. 1985. A very strong enhancer is located upstream of an immediate early gene of human cytomegalovirus. *Cell* 41:521-530.

24. Brodsky, F.M., W.F.Bodmer, and P.Parham. 1979. Characterization of a monoclonal anti-beta 2-microglobulin antibody and its use in the genetic and biochemical analysis of major histocompatibility antigens. *Eur. J. Immunol.* 9:536-545.

25. Browning, M.J., J.A.Madrigal, P.Krausa, H.Kowalski, C.E.Allsopp, A.M.Little, S.Turner, E.J.Adams, K.L.Arnett, W.F.Bodmer. 1995. The HLA-A,B,C genotype of the class I negative cell line Daudi reveals novel HLA-A and -B alleles. *Tissue Antigens* 45:177-187.

26. Bruggeman, F.J., W.C.van Heeswijk, F.C.Boogerd, and H.V.Westerhoff. 2000. Macromolecular intelligence in microorganisms. *Biol. Chem.* 381:965-972.

27. Burnet, F.M. 1959. The Clonal Selection Theory of Acquired Immunity. Cambride University Press.

28. Caplen, N.J., S.Parrish, F.Imani, A.Fire, and R.A.Morgan. 2001. Specific inhibition of gene expression by small double-stranded RNAs in invertebrate and vertebrate systems. *Proc. Natl. Acad. Sci. U. S. A* 98:9742-9747.

29. Cascio, P., C.Hilton, A.F.Kisselev, K.L.Rock, and A.L.Goldberg. 2001. 26S proteasomes and immunoproteasomes produce mainly N-extended versions of an antigenic peptide. *EMBO J* 20:2357-2366.

30. Castillo-Keller, M., P.Vuong, and R.Misra. 2006. Novel mechanism of Escherichia coli porin regulation. *J. Bacteriol.* 188:576-586.

31. Cerundolo, V., J.Alexander, K.Anderson, C.Lamb, P.Cresswell, A.McMichael, F.Gotch, and A.Townsend. 1990. Presentation of viral antigen controlled by a gene in the major histocompatibility complex. *Nature* 345:449-452.

24. Brodsky, F.M., W.F.Bodmer, and P.Parham. 1979. Characterization of a monoclonal anti-beta 2-microglobulin antibody and its use in the genetic and biochemical analysis of major histocompatibility antigens. *Eur. J. Immunol.* 9:536-545.

25. Browning, M.J., J.A.Madrigal, P.Krausa, H.Kowalski, C.E.Allsopp, A.M.Little, S.Turner, E.J.Adams, K.L.Arnett, W.F.Bodmer, and . 1995. The HLA-A,B,C genotype of the class I negative cell line Daudi reveals novel HLA-A and -B alleles. *Tissue Antigens* 45:177-187.

26. Bruggeman, F.J., W.C.van Heeswijk, F.C.Boogerd, and H.V.Westerhoff. 2000. Macromolecular intelligence in microorganisms. *Biol. Chem.* 381:965-972.

27. Burnet, F.M. 1959. The Clonal Selection Theory of Acquired Immunity. Cambride University Press.

28. Caplen, N.J., S.Parrish, F.Imani, A.Fire, and R.A.Morgan. 2001. Specific inhibition of gene expression by small double-stranded RNAs in invertebrate and vertebrate systems. *Proc. Natl. Acad. Sci. U. S. A* 98:9742-9747.

29. Cascio, P., C.Hilton, A.F.Kisselev, K.L.Rock, and A.L.Goldberg. 2001. 26S proteasomes and immunoproteasomes produce mainly N-extended versions of an antigenic peptide. *EMBO J* 20:2357-2366.

30. Castillo-Keller, M., P.Vuong, and R.Misra. 2006. Novel mechanism of Escherichia coli porin regulation. *J. Bacteriol.* 188:576-586.

31. Cerundolo, V., J.Alexander, K.Anderson, C.Lamb, P.Cresswell, A.McMichael, F.Gotch, and A.Townsend. 1990. Presentation of viral antigen controlled by a gene in the major histocompatibility complex. *Nature* 345:449-452.

32. Ciurea, A., P.Klenerman, L.Hunziker, E.Horvath, B.Odermatt, A.F.Ochsenbein, H.Hengartner, and R.M.Zinkernagel. 1999. Persistence of lymphocytic choriomeningitis virus at very low levels in immune mice. *Proc. Natl. Acad. Sci. U. S. A* 96:11964-11969.

33. Claman, H.N. and E.A.Chaperon. 1969. Immunologic complementation between thymus and marrow cells--a model for the two-cell theory of immunocompetence. *Transplant. Rev.* 1:92-113.

34. Copeman, J., N.Bangia, J.C.Cross, and P.Cresswell. 1998. Elucidation of the genetic basis of the antigen presentation defects in the mutant cell line .220 reveals polymorphism and alternative splicing of the tapasin gene. *Eur. J. Immunol.* 28:3783-3791.

35. Cordier, A.C. and S.M.Haumont. 1980. Development of thymus, parathyroids, and ultimo-branchial bodies in NMRI and nude mice. *Am. J. Anat.* 157:227-263.

36. Coscoy, L. and D.Ganem. 2003. PHD domains and E3 ubiquitin ligases: viruses make the connection. *Trends Cell Biol.* 13:7-12.

37. Coulie, P.G., V.Brichard, A.Van Pel, T.Wolfel, J.Schneider, C.Traversari, S.Mattei, E.De Plaen, C.Lurquin, J.P.Szikora, J.C.Renauld, and T.Boon. 1994. A new gene coding for a differentiation antigen recognized by autologous cytolytic T lymphocytes on HLA-A2 melanomas. *J. Exp. Med.* 180:35-42.

38. David, V., F.Hochstenbach, S.Rajagopalan, and M.B.Brenner. 1993. Interaction with newly synthesized and retained proteins in the endoplasmic reticulum suggests a chaperone function for human integral membrane protein IP90 (calnexin). *J Biol. Chem.* 268:9585-9592.

39. Davies, J.L., Y.Kawaguchi, S.T.Bennett, J.B.Copeman, H.J.Cordell, L.E.Pritchard, P.W.Reed, S.C.Gough, S.C.Jenkins, S.M.Palmer, and . 1994. A genome-wide search for human type 1 diabetes susceptibility genes. *Nature* 371:130-136.

40. De Silva, A.D., A.Boesteanu, R.Song, N.Nagy, E.Harhaj, C.V.Harding, and S.Joyce. 1999. Thermolabile H-2Kb molecules expressed by transporter associated with antigen processing-deficient RMA-S cells are occupied by low-affinity peptides. *J Immunol* 163:4413-4420.

41. Degen, E. and D.B.Williams. 1991. Participation of a novel 88-kD protein in the biogenesis of murine class I histocompatibility molecules. *J Cell Biol.* 112:1099-1115.

42. Deighton, C.M., D.J.Walker, I.D.Griffiths, and D.F.Roberts. 1989. The contribution of HLA to rheumatoid arthritis. *Clin. Genet.* 36:178-182.

43. Deverson, E.V., I.R.Gow, W.J.Coadwell, J.J.Monaco, G.W.Butcher, and J.C.Howard. 1990. MHC class II region encoding proteins related to the multidrug resistance family of transmembrane transporters. *Nature* 348:738-741.

44. Dexter, D.L., J.A.Barbosa, and P.Calabresi. 1979. N,N-dimethylformamide-induced alteration of cell culture characteristics and loss of tumorigenicity in cultured human colon carcinoma cells. *Cancer Res.* 39:1020-1025.

45. Dick, T.P., N.Bangia, D.R.Peaper, and P.Cresswell. 2002. Disulfide bond isomerization and the assembly of MHC class I-peptide complexes. *Immunity.* 16:87-98.

46. Diedrich, G., N.Bangia, M.Pan, and P.Cresswell. 2001. A role for calnexin in the assembly of the MHC class I loading complex in the endoplasmic reticulum. *J. Immunol.* 166:1703-1709.

47. Donze, O. and D.Picard. 2002. RNA interference in mammalian cells using siRNAs synthesized with T7 RNA polymerase. *Nucleic Acids Res.* 30:e46.

48. Dull, T., R.Zufferey, M.Kelly, R.J.Mandel, M.Nguyen, D.Trono, and L.Naldini. 1998. A third-generation lentivirus vector with a conditional packaging system. *J. Virol.* 72:8463-8471.

49. Dykxhoorn, D.M., C.D.Novina, and P.A.Sharp. 2003. Killing the messenger: short RNAs that silence gene expression. *Nat. Rev. Mol. Cell Biol.* 4:457-467.

50. Edelman, G.M. 1970. The structure and function of antibodies. *Sci. Am.* 223:34-42.

51. Elbashir, S.M., W.Lendeckel, and T.Tuschl. 2001a. RNA interference is mediated by 21- and 22-nucleotide RNAs. *Genes Dev.* 15:188-200.

52. Elbashir, S.M., J.Martinez, A.Patkaniowska, W.Lendeckel, and T.Tuschl. 2001b. Functional anatomy of siRNAs for mediating efficient RNAi in Drosophila melanogaster embryo lysate. *EMBO J.* 20:6877-6888.

53. Ellgaard, L. and A.Helenius. 2001. ER quality control: towards an understanding at the molecular level. *Current Opinions in Cell Biology*431-437.

54. Elliott, T. 1997. How does TAP associate with MHC class I molecules? *Immunol. Today* 18:375-379.

55. Emi, N., T.Friedmann, and J.K.Yee. 1991. Pseudotype formation of murine leukemia virus with the G protein of vesicular stomatitis virus. *J. Virol.* 65:1202-1207.

56. Falk, K., O.Rotzschke, S.Stevanovic, G.Jung, and H.G.Rammensee. 1991. Allele-specific motifs revealed by sequencing of self-peptides eluted from MHC molecules. *Nature* 351:290-296.

57. Farmery, M.R., S.Allen, A.J.Allen, and N.J.Bulleid. 2000. The role of ERp57 in disulfide bond formation during the assembly of major histocompatibility complex class I in a synchronized semipermeabilized cell translation system. *J Biol. Chem.* 275:14933-14938.

58. Fehling, H.J., A.Krotkova, C.Saint-Ruf, and H.von Boehmer. 1995. Crucial role of the pre-T-cell receptor alpha gene in development of alpha beta but not gamma delta T cells. *Nature* 375:795-798.

59. Fire, A., S.Xu, M.K.Montgomery, S.A.Kostas, S.E.Driver, and C.C.Mello. 1998. Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. *Nature* 391:806-811.

60. Flutter, B. and B.Gao. 2004. MHC class I antigen presentation--recently trimmed and well presented. *Cell Mol. Immunol.* 1:22-30.

61. Forsgren, A. and J.Sjoquist. 1966. "Protein A" from S. aureus. I. Pseudo-immune reaction with human gamma-globulin. *J. Immunol.* 97:822-827.

62. Fowlkes, B.J., L.Edison, B.J.Mathieson, and T.M.Chused. 1985. Early T lymphocytes. Differentiation in vivo of adult intrathymic precursor cells. *J. Exp. Med.* 162:802-822.

63. French, D.L., R.Laskov, and M.D.Scharff. 1989. The role of somatic hypermutation in the generation of antibody diversity. *Science* 244:1152-1157.

64. Gaczynska, M., K.L.Rock, and A.L.Goldberg. 1993. Gamma-interferon and expression of MHC genes regulate peptide hydrolysis by proteasomes. *Nature* 365:264-267.

65. Gagnon, E., S.Duclos, C.Rondeau, E.Chevet, P.H.Cameron, O.Steele-Mortimer, J.Paiement, J.J.Bergeron, and M.Desjardins. 2002. Endoplasmic reticulum-mediated phagocytosis is a mechanism of entry into macrophages. *Cell* 110:119-131.

66. Ganfornina, M.D. and D.Sanchez. 1999. Generation of evolutionary novelty by functional shift. *Bioessays* 21:432-439.

67. Gao, B., R.Adhikari, M.Howarth, K.Nakamura, M.C.Gold, A.B.Hill, R.Knee, M.Michalak, and T.Elliott. 2002. Assembly and antigen-presenting function of MHC class I molecules in cells lacking the ER chaperone calreticulin. *Immunity.* 16:99-109.

68. Gao, B., A.Williams, A.Sewell, and T.Elliott. 2004. Generation of a functional, soluble tapasin protein from an alternatively spliced mRNA. *Genes Immun.* 5:101-108.

69. Garbi, N., P.Tan, A.D.Diehl, B.J.Chambers, H.G.Ljunggren, F.Momburg, and G.J.Hammerling. 2000. Impaired immune responses and altered peptide repertoire in tapasin-deficient mice. *Nat. Immunol.* 1:234-238.

70. Garbi, N., S.Tanaka, F.Momburg, and G.J.Hammerling. 2006. Impaired assembly of the major histocompatibility complex class I peptide-loading complex in mice deficient in the oxidoreductase ERp57. *Nat. Immunol* 7:93-102.

71. Garbi, N., N.Tiwari, F.Momburg, and G.J.Hammerling. 2003. A major role for tapasin as a stabilizer of the TAP peptide transporter and consequences for MHC class I expression. *Eur. J Immunol* 33:264-273.

72. Garboczi, D.N., P.Ghosh, U.Utz, Q.R.Fan, W.E.Biddison, and D.C.Wiley. 1996. Structure of the complex between human T-cell receptor, viral peptide and HLA-A2. *Nature* 384:134-141.

73. Garcia, K.C., M.Degano, R.L.Stanfield, A.Brunmark, M.R.Jackson, P.A.Peterson, L.Teyton, and I.A.Wilson. 1996. An alphabeta T cell receptor structure at 2.5 A and its orientation in the TCR-MHC complex. *Science* 274:209-219.

74. Gilbert, H.F. 1997. Protein disulfide isomerase and assisted protein folding. *J. Biol. Chem.*29399-29402.

75. Gong, J.H., G.Maki, and H.G.Klingemann. 1994. Characterization of a human cell line (NK-92) with phenotypical and functional characteristics of activated natural killer cells. *Leukemia* 8:652-658.

76. Graham, F.L., J.Smiley, W.C.Russell, and R.Nairn. 1977. Characteristics of a human cell line transformed by DNA from human adenovirus type 5. *J. Gen. Virol.* 36:59-74.

77. Grandea, A.G., III, M.J.Androlewicz, R.S.Athwal, D.E.Geraghty, and T.Spies. 1995. Dependence of peptide binding by MHC class I molecules on their interaction with TAP. *Science* 270:105-108.

78. Grandea, A.G., III, T.N.Golovina, S.E.Hamilton, V.Sriram, T.Spies, R.R.Brutkiewicz, J.T.Harty, L.C.Eisenlohr, and L.Van Kaer. 2000. Impaired assembly yet normal trafficking of MHC class I molecules in Tapasin mutant mice. *Immunity.* 13:213-222.

79. Greenwood, R., Y.Shimizu, G.S.Sekhon, and R.DeMars. 1994. Novel allele-specific, post-translational reduction in HLA class I surface expression in a mutant human B cell line. *J. Immunol.* 153:5525-5536.

80. Grogan, J.L. and R.M.Locksley. 2002. T helper cell differentiation: on again, off again. *Curr. Opin. Immunol.* 14:366-372.

81. Guo, S. and K.J.Kemphues. 1995. par-1, a gene required for establishing polarity in C. elegans embryos, encodes a putative Ser/Thr kinase that is asymmetrically distributed. *Cell* 81:611-620.

82. Hamilton, A.J. and D.C.Baulcombe. 1999. A species of small antisense RNA in posttranscriptional gene silencing in plants. *Science* 286:950-952.

83. Hammond, C., I.Braakman, and A.Helenius. 1994. Role of N-linked oligosaccharide recognition, glucose trimming, and calnexin in glycoprotein folding and quality control. *Proc. Natl. Acad. Sci. U. S. A* 91:913-917.

84. Hansen, T.H., L.Lybarger, L.Yu, V.Mitaksov, and D.H.Fremont. 2005. Recognition of open conformers of classical MHC by chaperones and monoclonal antibodies. *Immunol. Rev.* 207:100-111.

85. Harter, C. and C.Reinhard. 2000. The secretory pathway from history to the state of the art. *Subcell. Biochem.* 34:1-38.

86. Hashimoto, C., K.L.Hudson, and K.V.Anderson. 1988. The Toll gene of Drosophila, required for dorsal-ventral embryonic polarity, appears to encode a transmembrane protein. *Cell* 52:269-279.

87. Herberg, J.A., J.Sgouros, T.Jones, J.Copeman, S.J.Humphray, D.Sheer, P.Cresswell, S.Beck, and J.Trowsdale. 1998. Genomic analysis of the Tapasin gene, located close to the TAP loci in the MHC. *Eur. J. Immunol.* 28:459-467.

88. Herman, J., B.P.van der, I.F.Luescher, S.Mandruzzato, P.Romero, J.Thonnard, K.Fleischhauer, T.Boon, and P.G.Coulie. 1996. A peptide encoded by the human MAGE3 gene and presented by HLA-B44 induces cytolytic T lymphocytes that recognize tumor cells expressing MAGE3. *Immunogenetics* 43:377-383.

89. Hewitt, E.W., S.S.Gupta, and P.J.Lehner. 2001. The human cytomegalovirus gene product US6 inhibits ATP binding by TAP. *EMBO J.* 20:387-396.

90. High, S., F.J.Lecomte, S.J.Russell, B.M.Abell, and J.D.Oliver. 2000. Glycoprotein folding in the endoplasmic reticulum: a tale of three chaperones? *FEBS Lett.* 476:38-41.

91. Hill, A.V., C.E.Allsopp, D.Kwiatkowski, N.M.Anstey, P.Twumasi, P.A.Rowe, S.Bennett, D.Brewster, A.J.McMichael, and B.M.Greenwood. 1991. Common west African HLA antigens are associated with protection from severe malaria. *Nature* 352:595-600.

92. Hirano, N., F.Shibasaki, R.Sakai, T.Tanaka, J.Nishida, Y.Yazaki, T.Takenawa, and H.Hirai. 1995. Molecular cloning of the human glucose regulated protein ERP57/GRP58, a thiol-dependent reductase. Identification of its secretory form and inducible expression by the oncogenic transformation. *Eur. J. Biochem.*336-342.

93. Horton, R., L.Wilming, V.Rand, R.C.Lovering, E.A.Bruford, V.K.Khodiyar, M.J.Lush, S.Povey, C.C.Talbot, Jr., M.W.Wright, H.M.Wain, J.Trowsdale, A.Ziegler, and S.Beck. 2004. Gene map of the extended human MHC. *Nat. Rev. Genet.* 5:889-899.

94. Houde, M., S.Bertholet, E.Gagnon, S.Brunet, G.Goyette, A.Laplante, M.F.Princiotta, P.Thibault, D.Sacks, and M.Desjardins. 2003. Phagosomes are competent organelles for antigen cross-presentation. *Nature* 425:402-406.

95. Howarth, M., A.Williams, A.B.Tolstrup, and T.Elliott. 2004. Tapasin enhances MHC class I peptide presentation according to peptide half-life. *Proc. Natl. Acad. Sci. U. S. A* 101:11737-11742.

96. Howell, D.N., P.E.Andreotti, J.R.Dawson, and P.Cresswell. 1985. Natural killing target antigens as inducers of interferon: studies with an immunoselected, natural killing-resistant human T lymphoblastoid cell line. *J. Immunol.* 134:971-976.

97. Huang, A.Y., A.T.Bruce, D.M.Pardoll, and H.I.Levitsky. 1996. In vivo cross-priming of MHC class I-restricted antigens requires the TAP transporter. *Immunity.* 4:349-355.

98. Huesmann, M., B.Scott, P.Kisielow, and H.von Boehmer. 1991. Kinetics and efficacy of positive selection in the thymus of normal and T cell receptor transgenic mice. *Cell* 66:533-540.

99. Hughes, E.A. and P.Cresswell. 1998. The thiol oxidoreductase ERp57 is a component of the MHC Class I peptide-loading complex. *Curr. Biol.*709-712.

100. Hughes, E.A., C.Hammond, and P.Cresswell. 1997. Misfolded major histocompatibility complex class I heavy chains are translocated into the cytoplasm and degraded by the proteasome. *Proc. Natl. Acad. Sci. U. S. A* 94:1896-1901.

101. Huppa, J.B. and H.L.Ploegh. 1998. The eS-Sence of -SH in the ER. *Cell* 92:145-148.

102. Invitrogen. Virapower Lentiviral Expression Systems. [K4950-00]. 2005.

103. Irwin, M.J., W.R.Heath, and L.A.Sherman. 1989. Species-restricted interactions between CD8 and the alpha 3 domain of class I influence the magnitude of the xenogeneic response. *J. Exp. Med.* 170:1091-1101.

104. Jainchill, J.L., S.A.Aaronson, and G.J.Todaro. 1969. Murine sarcoma and leukemia viruses: assay using clonal lines of contact-inhibited mouse cells. *J. Virol.* 4:549-553.

105. Janeway, C.A., T.Travers, M.Walport, and J.D.Capra. 1999. Immunobiology: The Immune System in Health and Disease. Harcourt Publishers Ltd..

106. Jensen, S., M.P.Gassama, and T.Heidmann. 1999. Taming of transposable elements by homology-dependent gene silencing. *Nat. Genet.* 21:209-212.

107. Justice, S.S., D.A.Hunstad, J.R.Harper, A.R.Duguay, J.S.Pinkner, J.Bann, C.Frieden, T.J.Silhavy, and S.J.Hultgren. 2005. Periplasmic peptidyl prolyl cis-trans isomerases are not essential for viability, but SurA is required for pilus biogenesis in Escherichia coli. *J. Bacteriol.* 187:7680-7686.

108. Kamogawa, Y., L.A.Minasi, S.R.Carding, K.Bottomly, and R.A.Flavell. 1993. The relationship of IL-4- and IFN gamma-producing T cells studied by lineage ablation of IL-4-producing cells. *Cell* 75:985-995.

109. Kappler, J.W., N.Roehm, and P.Marrack. 1987. T cell tolerance by clonal elimination in the thymus. *Cell* 49:273-280.

110. Kasahara, M., M.F.Flajnik, T.Ishibashi, and T.Natori. 1995. Evolution of the major histocompatibility complex: a current overview. *Transpl. Immunol.* 3:1-20.

111. Kaslow, R.A., M.Carrington, R.Apple, L.Park, A.Munoz, A.J.Saah, J.J.Goedert, C.Winkler, S.J.O'Brien, C.Rinaldo, R.Detels, W.Blattner, J.Phair, H.Erlich, and D.L.Mann. 1996. Influence of combinations of human major histocompatibility complex genes on the course of HIV-1 infection. *Nat. Med.* 2:405-411.

112. Kasper, M.R., J.F.Roeth, M.Williams, T.M.Filzen, R.I.Fleis, and K.L.Collins. 2005. HIV-1 Nef disrupts antigen presentation early in the secretory pathway. *J. Biol. Chem.* 280:12840-12848.

113. Kassiotis, G., R.Zamoyska, and B.Stockinger. 2003. Involvement of avidity for major histocompatibility complex in homeostasis of naive and memory T cells. *J. Exp. Med.* 197:1007-1016.

114. Kaye, J., M.L.Hsu, M.E.Sauron, S.C.Jameson, N.R.Gascoigne, and S.M.Hedrick. 1989. Selective development of CD4+ T cells in transgenic mice expressing a class II MHC-restricted antigen receptor. *Nature* 341:746-749.

115. Khan, S., R.de Giuli, G.Schmidtke, M.Bruns, M.Buchmeier, B.M.van den, and M.Groettrup. 2001. Cutting edge: neosynthesis is required for the presentation of a T cell epitope from a long-lived viral protein. *J. Immunol.* 167:4801-4804.

116. Kimura, M., A.Takatsuki, and I.Yamaguchi. 1994. Blasticidin S deaminase gene from Aspergillus terreus (BSD): a new drug resistance gene for transfection of mammalian cells. *Biochim. Biophys. Acta* 1219:653-659.

117. Klein, E., G.Klein, J.S.Nadkarni, J.J.Nadkarni, H.Wigzell, and P.Clifford. 1968. Surface IgM-kappa specificity on a Burkitt lymphoma cell in vivo and in derived culture lines. *Cancer Res.* 28:1300-1310.

118. Kloetzel, P.M. 2004. Generation of major histocompatibility complex class I antigens: functional interplay between proteasomes and TPPII. *Nat. Immunol.* 5:661-669.

119. Knittler, M.R., P.Alberts, E.V.Deverson, and J.C.Howard. 1999. Nucleotide binding by TAP mediates association with peptide and release of assembled MHC class I molecules. *Curr. Biol* 9:999-1008.

120. Komatsu, F. and M.Kajiwara. 1998. Relation of natural killer cell line NK-92-mediated cytolysis (NK-92-lysis) with the surface markers of major histocompatibility complex class I antigens, adhesion molecules, and Fas of target cells. *Oncol. Res.* 10:483-489.

121. Kovacsovics-Bankowski, M., K.Clark, B.Benacerraf, and K.L.Rock. 1993. Efficient major histocompatibility complex class I presentation of exogenous antigen upon phagocytosis by macrophages. *Proc. Natl. Acad. Sci. U. S. A* 90:4942-4946.

122. Kovacsovics-Bankowski, M. and K.L.Rock. 1995. A phagosome-to-cytosol pathway for exogenous antigens presented on MHC class I molecules. *Science* 267:243-246.

123. Kronvall, G., U.S.Seal, J.Finstad, and R.C.Williams, Jr. 1970. Phylogenetic insight into evolution of mammalian Fc fragment of gamma G globulin using staphylococcal protein A. *J. Immunol.* 104:140-147.

124. Kundig, T.M., M.F.Bachmann, S.Oehen, U.W.Hoffmann, J.J.Simard, C.P.Kalberer, H.Pircher, P.S.Ohashi, H.Hengartner, and R.M.Zinkernagel. 1996. On the role of antigen in maintaining cytotoxic T-cell memory. *Proc. Natl. Acad. Sci. U. S. A* 93:9716-9723.

125. Langone, J.J., M.D.Boyle, and T.Borsos. 1978. Studies on the interaction between protein A and immunoglobulin G. II. Composition and activity of complexes formed between protein A and IgG. *J. Immunol.* 121:333-338.

126. Lehner, P.J., M.J.Surman, and P.Cresswell. 1998. Soluble tapasin restores MHC class I expression and function in the tapasin-negative cell line .220. *Immunity.* 8:221-231.

127. Lewis, J.W., A.Neisig, J.Neefjes, and T.Elliott. 1996. Point mutations in the alpha 2 domain of HLA-A2.1 define a functionally relevant interaction with TAP. *Curr. Biol.* 6:873-883.

128. Lewis, J.W., A.Sewell, D.Price, and T.Elliott. 1998. HLA-A*0201 presents TAP-dependent peptide epitopes to cytotoxic T lymphocytes in the absence of tapasin. *Eur. J Immunol.* 28:3214-3220.

129. Li, S., K.M.Paulsson, S.Chen, H.O.Sjogren, and P.Wang. 2000. Tapasin is required for efficient peptide binding to transporter associated with antigen processing. *J. Biol. Chem.* 275:1581-1586.

130. Li, S., K.M.Paulsson, H.O.Sjogren, and P.Wang. 1999. Peptide-bound major histocompatibility complex class I molecules associate with tapasin before dissociation from transporter associated with antigen processing. *J. Biol. Chem.* 274:8649-8654.

131. Lie, W.R., N.B.Myers, J.M.Connolly, J.Gorka, D.R.Lee, and T.H.Hansen. 1991. The specific binding of peptide ligand to Ld class I major histocompatibility complex molecules determines their antigenic structure. *J. Exp. Med.* 173:449-459.

132. Lindquist, J.A., G.J.Hammerling, and J.Trowsdale. 2001. ER60/ERp57 forms disulfide-bonded intermediates with MHC class I heavy chain. *FASEB J*1448-1450.

133. Lindquist, J.A., O.N.Jensen, M.Mann, and G.J.Hammerling. 1998. ER-60, a chaperone with thiol-dependent reductase activity involved in MHC class I assembly. *EMBO J.*2186-2195.

134. Liu, Y. and C.A.Janeway, Jr. 1992. Cells that present both specific ligand and costimulatory activity are the most efficient inducers of clonal expansion of normal CD4 T cells. *Proc. Natl. Acad. Sci. U. S. A* 89:3845-3849.

135. Ljunggren, H.G. and K.Karre. 1990. In search of the 'missing self': MHC molecules and NK cell recognition. *Immunol. Today* 11:237-244.

136. Ljunggren, H.G., N.J.Stam, C.Ohlen, J.J.Neefjes, P.Hoglund, M.T.Heemels, J.Bastin, T.N.Schumacher, A.Townsend, K.Karre, and . 1990. Empty MHC class I molecules come out in the cold. *Nature* 346:476-480.

137. Lozzio, C.B. and B.B.Lozzio. 1975. Human chronic myelogenous leukemia cell-line with positive Philadelphia chromosome. *Blood* 45:321-334.

138. Luciw, P.A. 1996. Human Immunodeficiency Viruses and Their Replication. *In* Fields Virology. B.N.Fields, D.M.Knipe, P.M.Howley, R.M.Chanock, J.L.Melnick, T.P.Monath, B.Roizman, and S.E.Straus, editors. Lippincott-Raven Publishers, Philadelphia, PA. 1881-1975.

139. Malyguine, A.M., J.E.Scott, and J.R.Dawson. 1998. The role of calnexin in NK-target cell interaction. *Immunol. Lett.* 61:67-71.

140. Marguet, D., E.T.Spiliotis, T.Pentcheva, M.Lebowitz, J.Schneck, and M.Edidin. 1999. Lateral diffusion of GFP-tagged H2Ld molecules and of GFP-TAP1 reports on the assembly and retention of these molecules in the endoplasmic reticulum. *Immunity.* 11:231-240.

141. Martin, A.M., J.K.Kulski, C.Witt, P.Pontarotti, and F.T.Christiansen. 2002. Leukocyte Ig-like receptor complex (LRC) in mice and men. *Trends Immunol.* 23:81-88.

142. Medzhitov, R. and C.A.Janeway, Jr. 2002. Decoding the patterns of self and nonself by the innate immune system. *Science* 296:298-300.

143. Mesaeli, N., K.Nakamura, E.Zvaritch, P.Dickie, E.Dziak, K.H.Krause, M.Opas, D.H.MacLennan, and M.Michalak. 1999. Calreticulin is essential for cardiac development. *J. Cell Biol.* 144:857-868.

144. Meyer, A. 1998. Hox gene variation and evolution. *Nature* 391:225, 227-225, 228.

145. Miglino, O. and R.Walker. 2002. Genetic redundancy in evolving populations of simulated robots. *Artif. Life* 8:265-277.

146. MILLER, J.F. 1961. Immunological function of the thymus. *Lancet* 2:748-749.

147. Miretti, M.M., E.C.Walsh, X.Ke, M.Delgado, M.Griffiths, S.Hunt, J.Morrison, P.Whittaker, E.S.Lander, L.R.Cardon, D.R.Bentley, J.D.Rioux, S.Beck, and P.Deloukas. 2005. A high-resolution linkage-disequilibrium map of the human major histocompatibility complex and first generation of tag single-nucleotide polymorphisms. *Am. J. Hum. Genet.* 76:634-646.

148. Mombaerts, P., A.R.Clarke, M.A.Rudnicki, J.Iacomini, S.Itohara, J.J.Lafaille, L.Wang, Y.Ichikawa, R.Jaenisch, M.L.Hooper, and . 1992. Mutations in T-cell antigen receptor genes alpha and beta block thymocyte development at different stages. *Nature* 360:225-231.

149. Moretta, A. and L.Moretta. 1997. HLA class I specific inhibitory receptors. *Curr. Opin. Immunol.* 9:694-701.

150. Morgan, E.L. and W.O.Weigle. 1987. Biological activities residing in the Fc region of immunoglobulin. *Adv. Immunol.* 40:61-134.

151. Morrice, N.A. and S.J.Powis. 1998. A role for the thiol-dependent reductase ERp57 in the assembly of MHC class I molecules. *Curr. Biol.* 8:713-716.

152. Morris, P., J.Shaman, M.Attaya, M.Amaya, S.Goodman, C.Bergman, J.J.Monaco, and E.Mellins. 1994. An essential role for HLA-DM in antigen presentation by class II major histocompatibility molecules. *Nature* 368:551-554.

153. Morrison, L.A., A.E.Lukacher, V.L.Braciale, D.P.Fan, and T.J.Braciale. 1986. Differences in antigen presentation to MHC class I-and class II-restricted influenza virus-specific cytolytic T lymphocyte clones. *J. Exp. Med.* 163:903-921.

154. Mosmann, T.R. and R.L.Coffman. 1989. TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. *Annu. Rev. Immunol.* 7:145-173.

155. Napoli, C., C.Lemieux, and R.Jorgensen. 1990. Introduction of a Chimeric Chalcone Synthase Gene into Petunia Results in Reversible Co-Suppression of Homologous Genes in trans. *Plant Cell* 2:279-289.

156. Neumann, L., W.Kraas, S.Uebel, G.Jung, and R.Tampe. 1997. The active domain of the herpes simplex virus protein ICP47: a potent inhibitor of the transporter associated with antigen processing. *J. Mol. Biol.* 272:484-492.

157. Nussenzweig, M.C. and R.M.Steinman. 1980. Contribution of dendritic cells to stimulation of the murine syngeneic mixed leukocyte reaction. *J. Exp. Med.* 151:1196-1212.

158. O'Farrell, P.Z., H.M.Goodman, and P.H.O'Farrell. 1977. High resolution two-dimensional electrophoresis of basic as well as acidic proteins. *Cell* 12:1133-1141.

159. Ogawa, H., H.Miyazaki, and M.Kimura. 1971. Isolation and characterization of human skin lysozyme. *J. Invest Dermatol.* 57:111-116.

160. Oliver, J.D., H.L.Roderick, D.H.Llewellyn, and S.High. 1999. ERp57 functions as a subunit of specific complexes formed with the ER lectins calreticulin and calnexin. *Mol. Biol. Cell* 10:2573-2582.

161. Ortmann, B., M.J.Androlewicz, and P.Cresswell. 1994. MHC class I/beta 2-microglobulin complexes associate with TAP transporters before peptide binding. *Nature* 368:864-867.

162. Ortmann, B., J.Copeman, P.J.Lehner, B.Sadasivan, J.A.Herberg, A.G.Grandea, S.R.Riddell, R.Tampe, T.Spies, J.Trowsdale, and P.Cresswell. 1997. A critical role for tapasin in the assembly and function of multimeric MHC class I-TAP complexes. *Science* 277:1306-1309.

163. Owen, B.A. and L.R.Pease. 2001. Thermal stability of MHC class I-beta 2-microglobulin peptide complexes in the endoplasmic reticulum is determined by the peptide occupancy of the transporter associated with antigen processing complex. *J. Immunol.* 166:1740-1747.

164. Paquet, M.E., M.Cohen-Doyle, G.C.Shore, and D.B.Williams. 2004. Bap29/31 influences the intracellular traffic of MHC class I molecules. *J Immunol* 172:7548-7555.

165. Parham, P., C.J.Barnstable, and W.F.Bodmer. 1979. Use of a monoclonal antibody (W6/32) in structural studies of HLA-A,B,C, antigens. *J. Immunol.* 123:342-349.

166. Parham, P. and F.M.Brodsky. 1981. Partial purification and some properties of BB7.2. A cytotoxic monoclonal antibody with specificity for HLA-A2 and a variant of HLA-A28. *Hum. Immunol.* 3:277-299.

167. Park, B., Y.Kim, J.Shin, S.Lee, K.Cho, K.Fruh, S.Lee, and K.Ahn. 2004. Human cytomegalovirus inhibits tapasin-dependent peptide loading and optimization of the MHC class I peptide cargo for immune evasion. *Immunity.* 20:71-85.

168. Park, B., S.Lee, E.Kim, and K.Ahn. 2003. A single polymorphic residue within the Peptide-binding cleft of MHC class I molecules determines spectrum of tapasin dependence. *J Immunol.* 170:961-968.

169. Paulsson, K.M., P.O.Anderson, S.Chen, H.O.Sjogren, H.G.Ljunggren, P.Wang, and S.Li. 2001. Assembly of tapasin-associated MHC class I in the absence of the transporter associated with antigen processing (TAP). *Int. Immunol.* 13:23-29.

170. Paulsson, K.M., M.J.Kleijmeer, J.Griffith, M.Jevon, S.Chen, P.O.Anderson, H.O.Sjogren, S.Li, and P.Wang. 2002. Association of tapasin and COPI provides a mechanism for the retrograde transport of major histocompatibility complex (MHC) class I molecules from the Golgi complex to the endoplasmic reticulum. *J. Biol. Chem.* 277:18266-18271.

171. Paz, P., N.Brouwenstijn, R.Perry, and N.Shastri. 1999. Discrete proteolytic intermediates in the MHC class I antigen processing pathway and MHC I-dependent peptide trimming in the ER. *Immunity.* 11:241-251.

172. Peace-Brewer, A.L., L.G.Tussey, M.Matsui, G.Li, D.G.Quinn, and J.A.Frelinger. 1996. A point mutation in HLA-A*0201 results in failure to bind the TAP complex and to present virus-derived peptides to CTL. *Immunity.* 4:505-514.

173. Peaper, D.R., P.A.Wearsch, and P.Cresswell. 2005. Tapasin and ERp57 form a stable disulfide-linked dimer within the MHC class I peptide-loading complex. *EMBO J.* 24:3613-3623.

174. Peh, C.A., S.R.Burrows, M.Barnden, R.Khanna, P.Cresswell, D.J.Moss, and J.McCluskey. 1998. HLA-B27-restricted antigen presentation in the absence of tapasin reveals polymorphism in mechanisms of HLA class I peptide loading. *Immunity.* 8:531-542.

175. Petrie, H.T., P.Hugo, R.Scollay, and K.Shortman. 1990. Lineage relationships and developmental kinetics of immature thymocytes: CD3, CD4, and CD8 acquisition in vivo and in vitro. *J. Exp. Med.* 172:1583-1588.

176. Pfeifer, J.D., M.J.Wick, R.L.Roberts, K.Findlay, S.J.Normark, and C.V.Harding. 1993. Phagocytic processing of bacterial antigens for class I MHC presentation to T cells. *Nature* 361:359-362.

177. Powis, S.J., A.R.Townsend, E.V.Deverson, J.Bastin, G.W.Butcher, and J.C.Howard. 1991. Restoration of antigen presentation to the mutant cell line RMA-S by an MHC-linked transporter. *Nature* 354:528-531.

178. Prasad, S.A., J.W.Yewdell, A.Porgador, B.Sadasivan, P.Cresswell, and J.R.Bennink. 1998. Calnexin expression does not enhance the generation of MHC class I-peptide complexes. *Eur. J. Immunol.* 28:907-913.

179. Ratcliff, F.G., S.A.MacFarlane, and D.C.Baulcombe. 1999. Gene silencing without DNA. rna-mediated cross-protection between viruses. *Plant Cell* 11:1207-1216.

180. Reits, E.A., J.C.Vos, M.Gromme, and J.Neefjes. 2000. The major substrates for TAP in vivo are derived from newly synthesized proteins. *Nature* 404:774-778.

181. Rhim, J.S., H.Y.Cho, and R.J.Huebner. 1975. Non-producer human cells induced by murine sarcoma virus. *Int. J. Cancer* 15:23-29.

182. Rizzitello, A.E., J.R.Harper, and T.J.Silhavy. 2001. Genetic evidence for parallel pathways of chaperone activity in the periplasm of Escherichia coli. *J. Bacteriol.* 183:6794-6800.

183. Rock, K.L., L.Rothstein, S.Gamble, and C.Fleischacker. 1993. Characterization of antigen-presenting cells that present exogenous antigens in association with class I MHC molecules. *J. Immunol.* 150:438-446.

184. Rotter, J.I. and E.M.Landaw. 1984. Measuring the genetic contribution of a single locus to a multilocus disease. *Clin. Genet.* 26:529-542.

185. Rotzschke, O., K.Falk, K.Deres, H.Schild, M.Norda, J.Metzger, G.Jung, and H.G.Rammensee. 1990. Isolation and analysis of naturally processed viral peptides as recognized by cytotoxic T cells. *Nature* 348:252-254.

186. Rowen, L., B.F.Koop, and L.Hood. 1996. The complete 685-kilobase DNA sequence of the human beta T cell receptor locus. *Science* 272:1755-1762.

187. Sadasivan, B., P.J.Lehner, B.Ortmann, T.Spies, and P.Cresswell. 1996. Roles for calreticulin and a novel glycoprotein, tapasin, in the interaction of MHC class I molecules with TAP. *Immunity.* 5:103-114.

188. Sadasivan, B.K., A.Cariappa, G.L.Waneck, and P.Cresswell. 1995. Assembly, peptide loading, and transport of MHC class I molecules in a calnexin-negative cell line. *Cold Spring Harb. Symp. Quant. Biol.* 60:267-275.

189. Saint-Vis, B., I.Fugier-Vivier, C.Massacrier, C.Gaillard, B.Vanbervliet, S.Ait-Yahia, J.Banchereau, Y.J.Liu, S.Lebecque, and C.Caux. 1998. The cytokine profile expressed by human dendritic cells is dependent on cell subtype and mode of activation. *J. Immunol.* 160:1666-1676.

190. Sakaguchi, S., M.Toda, M.Asano, M.Itoh, S.S.Morse, and N.Sakaguchi. 1996. T cell-mediated maintenance of natural self-tolerance: its breakdown as a possible cause of various autoimmune diseases. *J. Autoimmun.* 9:211-220.

191. Salter, R.D. and P.Cresswell. 1986. Impaired assembly and transport of HLA-A and -B antigens in a mutant TxB cell hybrid. *EMBO J.* 5:943-949.

192. Salter, R.D., D.N.Howell, and P.Cresswell. 1985. Genes regulating HLA class I antigen expression in T-B lymphoblast hybrids. *Immunogenetics* 21:235-246.

193. Saric, T., S.C.Chang, A.Hattori, I.A.York, S.Markant, K.L.Rock, M.Tsujimoto, and A.L.Goldberg. 2002. An IFN-gamma-induced aminopeptidase in the ER, ERAP1, trims precursors to MHC class I-presented peptides. *Nat. Immunol* 3:1169-1176.

194. Scott, J.E. and J.R.Dawson. 1995. MHC class I expression and transport in a calnexin-deficient cell line. *J Immunol* 155:143-148.

195. Selsted, M.E., S.S.Harwig, T.Ganz, J.W.Schilling, and R.I.Lehrer. 1985. Primary structures of three human neutrophil defensins. *J. Clin. Invest* 76:1436-1439.

196. Seong, R.H., C.A.Clayberger, A.M.Krensky, and J.R.Parnes. 1988. Rescue of Daudi cell HLA expression by transfection of the mouse beta 2-microglobulin gene. *J. Exp. Med.* 167:288-299.

197. Serwold, T., F.Gonzalez, J.Kim, R.Jacob, and N.Shastri. 2002. ERAAP customizes peptides for MHC class I molecules in the endoplasmic reticulum. *Nature* 419:480-483.

198. Sesma, L., B.Galocha, M.Vazquez, A.W.Purcell, M.Marcilla, J.McCluskey, and J.A.Lopez de Castro. 2005. Qualitative and quantitative differences in peptides bound to HLA-B27 in the presence of mouse versus human tapasin define a role for tapasin as a size-dependent peptide editor. *J. Immunol.* 174:7833-7844.

199. Shen, L., L.J.Sigal, M.Boes, and K.L.Rock. 2004. Important role of cathepsin S in generating peptides for TAP-independent MHC class I crosspresentation in vivo. *Immunity.* 21:155-165.

200. Spee, P. and J.Neefjes. 1997. TAP-translocated peptides specifically bind proteins in the endoplasmic reticulum, including gp96, protein disulfide isomerase and calreticulin. *Eur. J Immunol* 27:2441-2449.

201. Spies, T., M.Bresnahan, S.Bahram, D.Arnold, G.Blanck, E.Mellins, D.Pious, and R.DeMars. 1990. A gene in the human major histocompatibility complex class II region controlling the class I antigen presentation pathway. *Nature* 348:744-747.

202. Spies, T., V.Cerundolo, M.Colonna, P.Cresswell, A.Townsend, and R.DeMars. 1992. Presentation of viral antigen by MHC class I molecules is dependent on a putative peptide transporter heterodimer. *Nature* 355:644-646.

203. Spies, T. and R.DeMars. 1991. Restored expression of major histocompatibility class I molecules by gene transfer of a putative peptide transporter. *Nature* 351:323-324.

204. Spiliotis, E.T., H.Manley, M.Osorio, M.C.Zuniga, and M.Edidin. 2000. Selective export of MHC class I molecules from the ER after their dissociation from TAP. *Immunity.* 13:841-851.

205. Stam, N.J., H.Spits, and H.L.Ploegh. 1986. Monoclonal antibodies raised against denatured HLA-B locus heavy chains permit biochemical characterization of certain HLA-C locus products. *J. Immunol.* 137:2299-2306.

206. Stenzel, A., T.Lu, W.A.Koch, J.Hampe, S.M.Guenther, F.M.De La Vega, M.Krawczak, and S.Schreiber. 2004. Patterns of linkage disequilibrium in the MHC region on human chromosome 6p. *Hum. Genet.* 114:377-385.

207. Suh, W.K., M.F.Cohen-Doyle, K.Fruh, K.Wang, P.A.Peterson, and D.B.Williams. 1994. Interaction of MHC class I molecules with the transporter associated with antigen processing. *Science* 264:1322-1326.

208. Suh, W.K., E.K.Mitchell, Y.Yang, P.A.Peterson, G.L.Waneck, and D.B.Williams. 1996. MHC class I molecules form ternary complexes with calnexin and TAP and undergo peptide-regulated interaction with TAP via their extracellular domains. *J. Exp. Med.* 184:337-348.

209. Super, M., S.Thiel, J.Lu, R.J.Levinsky, and M.W.Turner. 1989. Association of low levels of mannan-binding protein with a common defect of opsonisation. *Lancet* 2:1236-1239.

210. Suvas, S., U.Kumaraguru, C.D.Pack, S.Lee, and B.T.Rouse. 2003. CD4+CD25+ T cells regulate virus-specific primary and memory CD8+ T cell responses. *J. Exp. Med.* 198:889-901.

211. TAKEUCHI, S., K.HIRAYAMA, K.UEDA, H.SAKAI, and H.YONEHARA. 1958. Blasticidin S, a new antibiotic. *J. Antibiot. (Tokyo)* 11:1-5.

212. Tan, L., M.H.Andersen, T.Elliott, and J.S.Haurum. 1997. An improved assembly assay for peptide binding to HLA-B*2705 and H- 2K(k) class I MHC molecules. *J Immunol. Methods* 209:25-36.

213. Tan, P., H.Kropshofer, O.Mandelboim, N.Bulbuc, G.J.Hammerling, and F.Momburg. 2002. Recruitment of MHC class I molecules by tapasin into the transporter associated with antigen processing-associated complex is essential for optimal peptide loading. *J Immunol* 168:1950-1960.

214. Tay, C.H., L.Y.Yu, V.Kumar, L.Mason, J.R.Ortaldo, and R.M.Welsh. 1999. The role of LY49 NK cell subsets in the regulation of murine cytomegalovirus infections. *J. Immunol.* 162:718-726.

215. The MHC Sequencing Consortium. 1999. Complete sequence and gene map of a human major histocompatibility complex. The MHC sequencing consortium. *Nature* 401:921-923.

216. Tonegawa, S. 1983. Somatic generation of antibody diversity. *Nature* 302:575-581.

217. Tortorella, D., B.E.Gewurz, M.H.Furman, D.J.Schust, and H.L.Ploegh. 2000. Viral subversion of the immune system. *Annu. Rev. Immunol.* 18:861-926.

218. Tourdot, S., M.Nejmeddine, S.J.Powis, and K.G.Gould. 2005. Different MHC class I heavy chains compete with each other for folding independently of beta 2-microglobulin and peptide. *J. Immunol.* 174:925-933.

219. Townsend, A.R., J.Rothbard, F.M.Gotch, G.Bahadur, D.Wraith, and A.J.McMichael. 1986. The epitopes of influenza nucleoprotein recognized by cytotoxic T lymphocytes can be defined with short synthetic peptides. *Cell* 44:959-968.

220. Townsend, A.R. and J.J.Skehel. 1982. Influenza A specific cytotoxic T-cell clones that do not recognize viral glycoproteins. *Nature* 300:655-657.

221. Traherne, J.A., R.Horton, A.N.Roberts, M.M.Miretti, M.E.Hurles, C.A.Stewart, J.L.Ashurst, A.M.Atrazhev, P.Coggill, S.Palmer, J.Almeida, S.Sims, L.G.Wilming, J.Rogers, P.J.de Jong, M.Carrington, J.F.Elliott, S.Sawcer, J.A.Todd, J.Trowsdale, and S.Beck. 2006. Genetic Analysis of Completely Sequenced Disease-Associated MHC Haplotypes Identifies Shuffling of Segments in Recent Human History. *PLoS. Genet.* 2:e9.

222. Trowsdale, J., I.Hanson, I.Mockridge, S.Beck, A.Townsend, and A.Kelly. 1990. Sequences encoded in the class II region of the MHC related to the 'ABC' superfamily of transporters. *Nature* 348:741-744.

223. Udono, H. and P.K.Srivastava. 1993. Heat shock protein 70-associated peptides elicit specific cancer immunity. *J. Exp. Med.* 178:1391-1396.

224. Uger, R.A., S.M.Chan, and B.H.Barber. 1999. Covalent linkage to beta2-microglobulin enhances the MHC stability and antigenicity of suboptimal CTL epitopes. *J. Immunol.* 162:6024-6028.

225. Urade, R., T.Oda, H.Ito, T.Moriyama, S.Utsumi, and M.Kito. 1997. Functions of characteristic Cys-Gly-His-Cys (CGHC) and Gln-Glu-Asp-Leu (QEDL) motifs of microsomal ER-60 protease. *J. Biochem. (Tokyo)* 122:834-842.

226. van Bleek, G.M. and S.G.Nathenson. 1991. The structure of the antigen-binding groove of major histocompatibility complex class I molecules determines specific selection of self-peptides. *Proc. Natl. Acad. Sci. U. S. A* 88:11032-11036.

227. van der Krol, A.R., L.A.Mur, M.Beld, J.N.Mol, and A.R.Stuitje. 1990. Flavonoid genes in petunia: addition of a limited number of gene copies may lead to a suppression of gene expression. *Plant Cell* 2:291-299.

228. van der Slik, A.R., B.P.Koeleman, W.Verduijn, G.J.Bruining, B.O.Roep, and M.J.Giphart. 2003. KIR in type 1 diabetes: disparate distribution of activating and inhibitory natural killer cell receptors in patients versus HLA-matched control subjects. *Diabetes* 52:2639-2642.

229. van Leeuwen, J.E. and K.P.Kearse. 1996. Deglucosylation of N-linked glycans is an important step in the dissociation of calreticulin-class I-TAP complexes. *Proc. Natl. Acad. Sci. U. S. A* 93:13997-14001.

230. Villadangos, J.A., P.Schnorrer, and N.S.Wilson. 2005. Control of MHC class II antigen presentation in dendritic cells: a balance between creative and destructive forces. *Immunol. Rev.* 207:191-205.

231. Wang, Z., X.Z.Hu, R.J.Tatake, S.Y.Yang, R.A.Zeff, and S.Ferrone. 1993. Differential effect of human and mouse beta 2-microglobulin on the induction and the antigenic profile of endogenous HLA-A and -B antigens synthesized by beta 2-microglobulin gene-null FO-1 melanoma cells. *Cancer Res.* 53:4303-4309.

232. West, W.H., G.B.Cannon, H.D.Kay, G.D.Bonnard, and R.B.Herberman. 1977. Natural cytotoxic reactivity of human lymphocytes against a myeloid cell line: characterization of effector cells. *J. Immunol.* 118:355-361.

233. Wieland, F.T., M.L.Gleason, T.A.Serafini, and J.E.Rothman. 1987. The rate of bulk flow from the endoplasmic reticulum to the cell surface. *Cell* 50:289-300.

234. Wiertz, E.J., T.R.Jones, L.Sun, M.Bogyo, H.J.Geuze, and H.L.Ploegh. 1996. The human cytomegalovirus US11 gene product dislocates MHC class I heavy chains from the endoplasmic reticulum to the cytosol. *Cell* 84:769-779.

235. Williams, A.P., C.A.Peh, A.W.Purcell, J.McCluskey, and T.Elliott. 2002. Optimization of the MHC class I peptide cargo is dependent on tapasin. *Immunity.* 16:509-520.

236. Williams, D.B. 1995. Calnexin leads glycoproteins into the fold. *Glycoconj. J.* 12:iii-iiv.

237. Williams, D.B. 2006. Beyond lectins: the calnexin/calreticulin chaperone system of the endoplasmic reticulum. *J. Cell Sci.* 119:615-623.

238. Williams, D.B., B.H.Barber, R.A.Flavell, and H.Allen. 1989. Role of beta 2-microglobulin in the intracellular transport and surface expression of murine class I histocompatibility molecules. *J. Immunol.* 142:2796-2806.

239. Yee, C., J.A.Thompson, D.Byrd, S.R.Riddell, P.Roche, E.Celis, and P.D.Greenberg. 2002. Adoptive T cell therapy using antigen-specific CD8+ T cell clones for the treatment of patients with metastatic melanoma: in vivo persistence, migration, and antitumor effect of transferred T cells. *Proc. Natl. Acad. Sci. U. S. A* 99:16168-16173.

240. Yewdell, J.W. 2005. The seven dirty little secrets of major histocompatibility complex class I antigen processing. *Immunol. Rev.* 207:8-18.

241. York, I.A., S.C.Chang, T.Saric, J.A.Keys, J.M.Favreau, A.L.Goldberg, and K.L.Rock. 2002. The ER aminopeptidase ERAP1 enhances or limits antigen presentation by trimming epitopes to 8-9 residues. *Nat. Immunol* 3:1177-1184.

242. York, I.A., E.P.Grant, A.M.Dahl, and K.L.Rock. 2005. A mutant cell with a novel defect in MHC class I quality control. *J. Immunol.* 174:6839-6846.

243. Yu, Y.Y., N.Netuschil, L.Lybarger, J.M.Connolly, and T.H.Hansen. 2002. Cutting edge: single-chain trimers of MHC class I molecules form stable structures that potently stimulate antigen-specific T cells and B cells. *J. Immunol.* 168:3145-3149.

244. Yu, Y.Y., H.R.Turnquist, N.B.Myers, G.K.Balendiran, T.H.Hansen, and J.C.Solheim. 1999. An extensive region of an MHC class I alpha 2 domain loop influences interaction with the assembly complex. *J. Immunol.* 163:4427-4433.

245. Zarling, A.L., C.J.Luckey, J.A.Marto, F.M.White, C.J.Brame, A.M.Evans, P.J.Lehner, P.Cresswell, J.Shabanowitz, D.F.Hunt, and V.H.Engelhard. 2003. Tapasin is a facilitator, not an editor, of class I MHC peptide binding. *J Immunol* 171:5287-5295.

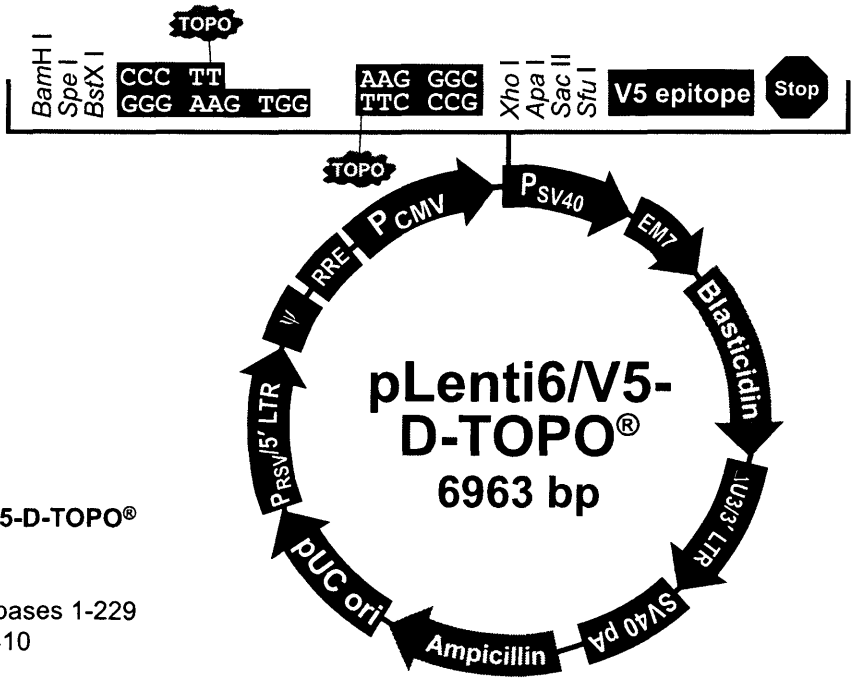
246. Zhang, Q. and R.D.Salter. 1998. Distinct patterns of folding and interactions with calnexin and calreticulin in human class I MHC proteins with altered N-glycosylation. *J. Immunol.* 160:831-837.

247. Zhang, Q., M.Tector, and R.D.Salter. 1995. Calnexin recognizes carbohydrate and protein determinants of class I major histocompatibility complex molecules. *J Biol. Chem.* 270:3944-3948.

248. Zinkernagel, R.M. 2002. On cross-priming of MHC class I-specific CTL: rule or exception? *Eur. J. Immunol.* 32:2385-2392.

249. Zinkernagel, R.M. and P.C.Doherty. 1974. Restriction of in vitro T cell-mediated cytotoxicity in lymphocytic choriomeningitis within a syngeneic or semiallogeneic system. *Nature* 248:701-702.

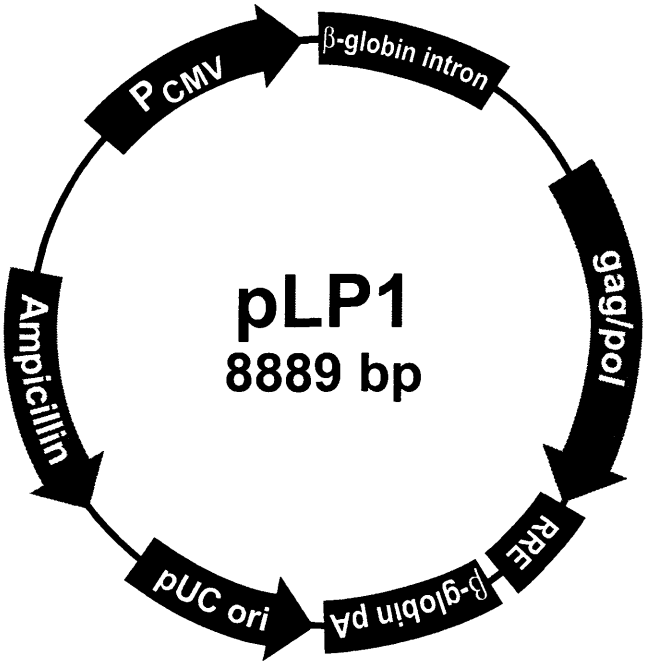
Appendix I



Comments for pLenti6/V5-D-TOPO®
6963 nucleotides

RSV enhancer/promoter: bases 1-229
HIV-1 5′ LTR: bases 230-410
5′ splice donor: base 520
HIV-1 psi (ψ) packaging sequence: bases 521-565
HIV-1 Rev response element (RRE): bases 1075-1308
3′ splice acceptor: base 1656
3′ splice acceptor: base 1684
CMV promoter: bases 1809-2392
CMV forward priming site: bases 2274-2294
Directional TOPO® site: bases 2431-2444
V5 epitope: bases 2473-2514
V5(C-term) reverse priming site: bases 2482-2502
SV40 early promoter and origin: bases 2569-2877
EM7 promoter: bases 2932-2998
Blasticidin resistance gene: bases 2999-3397
ΔU3/HIV-1 3′ LTR: bases 3484-3717
ΔU3: bases 3484-3536
Truncated HIV-1 3′ LTR: bases 3537-3717
SV40 polyadenylation signal: bases 3789-3920
bla promoter: bases 4779-4877
Ampicillin (*bla*) resistance gene: bases 4878-5738
pUC origin: bases 5883-6556

invitrogen™



Comments for pLP1
8889 nucleotides

CMV promoter: bases 1-747
TATA box: bases 648-651
Human β-globin intron: bases 880-1320
HIV-1 gag/pol sequences: bases 1355-5661
gag coding sequence: bases 1355-2857
gag/pol frameshift: base 2650
pol coding sequence: bases 2650-5661
HIV-1 Rev response element (RRE): bases 5686-5919
Human β-globin polyadenylation signal: bases 6072-6837
pUC origin: bases 6995-7668 (C)
Ampicillin (*bla*) resistance gene: bases 7813-8673 (C)
bla promoter: bases 8674-8772 (C)

C=complementary strand

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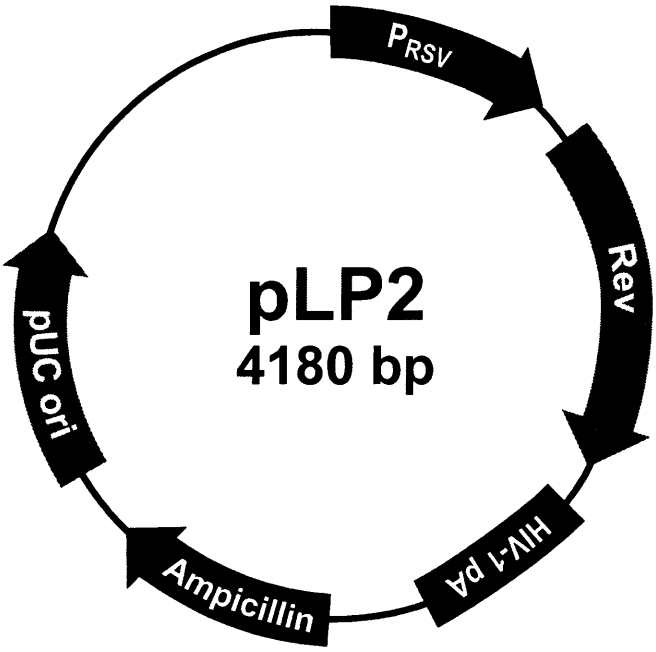

Comments for pLP2
4180 nucleotides

RSV enhancer/promoter: bases 1-271
TATA box: bases 200-207
Transcription initiation site: base 229
RSV UTR: bases 230-271
HIV-1 Rev ORF: bases 391-741
HIV-1 LTR polyadenylation signal: bases 850-971
bla promoter: bases 1916-2014
Ampicillin (*bla*) resistance gene: bases 2015-2875
pUC origin: bases 3020-3693

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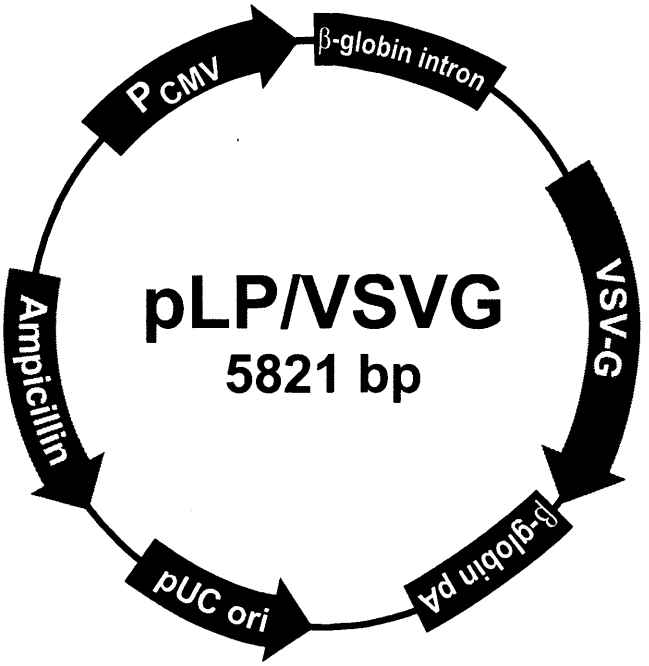
life technologies



Comments for pLP/VSVG
5821 nucleotides

CMV promoter: bases 1-747
TATA box: bases 648-651
Human β-globin intron: bases 880-1320
VSV G glycoprotein (VSV-G): bases 1346-2881
Human β-globin polyadenylation signal: bases 3004-3769
pUC origin: bases 3927-4600 (C)
Ampicillin (*bla*) resistance gene: bases 4745-5605 (C)
bla promoter: bases 5606-5704 (C)

C=complementary strand

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