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Functional Role of Calreticulin in MHC Class I Antigen Presentation

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Abstract

MHC class I antigen presentation to cytotoxic T cells (CTLs) is critically important to maintain immunity against pathogens and tumour-transformed cells. In this conventional class I pathway, peptides generated from endogenous proteins in the cytosol by proteasomes are transported into the ER lumen and loaded onto class I heavy chain-β2microglobulin heterodimers with the assistance of peptide loading complex (PLC) to form MHC class I complexes, which then traffic to the cell surface for the inspection by CTLs. The PLC contains transporter associated with antigen processing (TAP), tapasin, calreticulin (CRT), ERp57, and possibly calnexin. CRT is a glycoprotein chaperone and can be divided into three structural and functional domains: N-terminus domain, central P domain and C-terminus domain. CRT plays a critical role in MHC Class I antigen processing and presentation and can elicit peptide-specific CD8+ T cell responses against tumours when exogenously administered with peptides. However, how CRT contributes to class I antigen presentation and the mechanism of its adjuvant effect in anti-tumour responses remain to be elucidated.

To study the conventional MHC class I pathway, the protein antigen of interest has to be expressed inside cells. First, a novel method was described to deliver protein antigen directly into the cell cytosol. Mouse fibroblasts, lymphoma cell line RMA and its mutant counterpart cell line RMA-S were permeabilized with streptolysin O (SLO) first and chicken ovalbumin (OVA) was successfully introduced into both adherent and suspension cells. The OVA was properly processed and OVA-derived peptide $OVA_{258-265}$ (or SIINFEKL) was successfully presented via MHC class I molecule (K^b) to SIINFEKL- K^b specific T cell hybridoma-B3Z, while in RMA-S cell with TAP-2 deficient, the defect for MHC class I antigen processing was demonstrated.

Next, using the SLO antigen delivery system, OVA was introduced into the cytoplasm of both CRT deficient mouse fibroblasts (K42) and CRT competent cells (K41), and K42 cells transfected with full length human CRT to investigate how CRT contributes to class I antigen presentation and the mechanism of its adjuvant effect in anti-tumour responses.

It was showed that reduced class I expression in CRT deficient cells can be restored by the direct delivery of peptides into the ER or by incubation at low temperature. CRT deficient cells exhibit a TAP deficient phenotype in terms of class I assembly, without loss of TAP expression or functionality. Furthermore, a higher concentration of antigen in the cytosol is required for specific T cell stimulation in CRT deficient cells than that in wild type cells. The data indicates that CRT has a functional role in the maintenance of the low peptide concentration threshold required in the ER for efficient antigen presentation to T cells.

Finally, to investigate the functional role of CRT related to its structure and the possible mechanism of CRT lowering the peptide concentration threshold required in the ER for efficient class I antigen presentation, I have made a series of constructs containing P-, NP-, PC-domains and full length CRT and established their expression in both K42 and K41 cells. While full length CRT restores MHC class I surface expression on K42 cells to the levels seen in the wild-type K41 cells, the expression of P-, NP-, PC-domains of CRT down-regulates MHC class I expression in K42 cells. In contrast, K41 cells transfected with truncated CRT exhibits a normal class I surface expression, indicating that disruption to MHC class I assembly only happens in the absence of CRT. In further experiments, I have demonstrated that the defect is due to empty or peptide suboptimally loaded class I complex while direct delivery of peptides into the ER or incubated at 26 where class I stabilized the class I surface expression recover to nearly normal level. After excluding several possible defects during antigen processing and presentation, such as negative effects on expression of components of PLP, TAP function, class I assembly, I propose that calnexin may compensate for the loss of CRT function in cells without CRT. The expression of truncated forms of CRT in CRT deficient cells might block the redundant function of calnexin, leading to further reduced cell surface MHC class I expression.

Figures and Tables

Figures

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Abbreviations

β_2m	β-2 microglobulin
βgal	β- galactosidase protein
ABC	Antigen binding cleft
APC	Antigen presenting cell
ATP	Adenosine tri phosphate
Bap	B cell-associated protein
BFA	Brefeldin A
BiP	Immunoglobulin binding protein
bp	base pairs
CCT	Chaperonin-containing TCP-1
CD	Cluster of differentiation
cDNA	complementary deoxyribonucleic Acid
CMV	Cytomegalovirus
CNX	calnexin
COPI	Coat protein I
COPII	Coat protein II
CPRG	Chlorophenol red β-galactoside
CRP	C-reactive protein
CRT	Calreticulin
CTL	Cytotoxic T Lymphocyte
DC	Dendritic cell
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic Acid
dNTP	Deoxy-nucleotide triphosphate
DRiPs	Defective ribosomal products
dsRNA	Double stranded RNA
EDTA	Ethylene diaminetetraacetic acid
eGFP	enhanced Green Fluorescent Protein
ER	Endoplasmic Reticulum

ERAAP	ER aminopeptidase associated with antigen processing
ERAD	ER-associated degradation
ERp57	Endoplasmic Reticulum protein 57 kDa
ES	Embryonic stem
EtBr	Ethidium Bromide
E:T	Effector:Target
FACS	Fluorescence activated cell sorting
Fas-L	Ligand for Fas
FCS	Foetal calf serum
Fig	Figure
FITC	Fluorescein isothiocyanate
g	Gravity
G	Guanine
GFP	Green Fluorescent Protein
GT	Glycoprotein glucosyltransferase
HC	Heavy chain
HIV	Human Immunodeficiency Virus
HLA	Human Leukocyte Antigen
HRP	Horse radish peroxidase
Hsps	Heat shock proteins
HSV	Herpes simplex virus
H-2	histocompatibility-2
IFNγ	Interferon gamma
Ig	Immunoglobulin
IP	Immunoprecipitation
kDa	Kilodalton
Lac Z	Gene coding for β- galactosidase protein
LAP	Leucine aminopeptidase
LB	Luria Broth
LPS	Lipopolysaccharide
M	Molar

mAb	monoclonal antibody
MHC	Major Histocompatibility Complex
ml	millilitre
MOP	Multiplicity of infection
MP	Matrix protein
MW	Molecular weight
mRNA	Messenger ribonucleic acid
NBD	Nucleotide-binding domain
NK	Natural killer cell
NKR	calnexin deficient cell line
NP	Nucleoprotein
OVA	Ovalbumin
P/S/glu	Penicillin/ streptomycin/ glutamine
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline/ Tween
PCR	Polymerase Chain Reaction
PDI	Protein disulfide isomerase
PE	R-Phycoerythrin
PI	Propidium iodide
PLC	Peptide-loading complex
pMHC	peptide-MHC
PSA	Puromycin-sensitive aminopeptidase
RNA	Ribonucleic acid
RNAi	RNA interference
rpm	revolutions per minute
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SLO	Streptolysin-O
siRNA	Small interfering RNA
T	Thymidine
TAE	Tris/Acetate/EDTA

TAP	Transporter associated with antigen processing
TBE	Tris/borate/EDTA
TCP-1	Tailless complex polypeptide-1
TCR	T cell receptor
Th	T helper cell
TLR	Toll-like Receptor
TMD	Transmembrane domain
TNF α	Tumour necrosis factor α
TOP	Thimet oligopeptidase
TOPO	Topo-isomerase
Tpn	Tapasin (TAP associated protein)
TPP II	Tripeptidyl peptidase II
Treg	T regulatory cell
TRiC	Tailless complex polypeptide-1 ring complex
tRNA	Transfer RNA
U	International Units
UGGT	CRT/UDP glucosyl glycoprotein transferase
μl	Microlitre
UPR	Unfolded protein response

Chapter 1 Introduction

Humoral (antibody-mediated) and cellular (T cell-mediated) immunity are the two main lines of defence used by higher organisms to combat pathogens. While antibodies recognize free antigens, T cells distinguish foreign from self-materials through presentation of antigen fragments by cell surface major histocompatibility complex (MHC) molecules. There are two main types of T cells, CD4 T cells and CD8 T cells. Initially it was believed that CD4 T cells recognize antigenic peptide derived from exogenous proteins that have been internalised, processed and presented by class II MHC molecules, while CD8 T cells recognize antigen fragment derived from endogenous proteins and presented by MHC class I molecules(Townsend et al., 1985). However, there are some exceptions that are in contradiction to the classical view, which will be discussed later when appropriate.

$CD8^+$ T cells represent an important branch of immune system in non-self immune recognition. Each $CD8^+$ T lymphocyte produced in the thymus carries a different T cell receptor (TCR) capable of recognising peptide/MHC class I complexes. Once stimulated by interacting with specific peptide bound MHC class I molecules through their receptors, $CD8^+$ T cells will be activated and, with the help from CD4 T cells, they will proliferate and differentiate into cytotoxic T cells (CTLs), which will take action against the target cells. In this way CTLs can eradicate cells carrying foreign, mutated or in some cases over-expressed proteins(Shastri et al., 2002). How proteins are processed and bound to MHC class I molecule capable of interacting with T cell receptors has been and still is one of core themes in modern immunology.

1.1 MHC class I complex

The term major histocompatibility complex (MHC) actually refers to a region of DNA spanning some 4Mbp (base pair) and containing over 100 genes. It is located on chromosome 6 in the human and chromosome 17 in the mouse. Each species has a unique name for its MHC. In human the MHC is called human leukocyte antigen (HLA), and in mouse it is called histocompatibility-2 (H-2).

The MHC class I complex is made up of a 45 kDa transmembrane heavy chain (HC) glycoprotein (also called α-chain), a 12 kDa soluble light chain protein called β2-microglobulin (β2m), and a short peptide of 8-10 residues derived from endogenous proteins as well as virus- and tumor-specific proteins. The α-chain has three domains, called α1, α2 and α_3. The membrane-distal α_1 and α_2 domains are highly polymorphic. Diversity in these domains is very important in that these two domains form the antigen binding cleft (ABC) of the MHC class I molecule (Fig 1.1). The sides of the ABC are formed by α_1 and α_2, while the floor of ABC is comprised of eight anti-parallel β sheets(Bjorkman et al., 1987). The α_3 domain is situated under the α_1 and α_2 domains and anchors the class I HC to the cell membrane. The β2m non-covalently associates with class I HC and is proximal to the cell membrane. The antigenic peptide binds to the ABC in an extended conformation and interacts with MHC residues along the binding site with relatively low specificity but high stability(Rammensee et al., 1995). The interactions are important to maintain the stability of MHC class I molecules assembled(Bouvier and Wiley, 1998; Paulsson et al., 2001). For example, the affinity between HC and β2m is enhanced at least 10-fold in the presence of peptide ligand(Elliott et al., 1991), and upon peptide dissociation, there is loss of both β2m and conformation-sensitive epitopes within the HC(Smith et al., 1992; Smith et al., 1993).

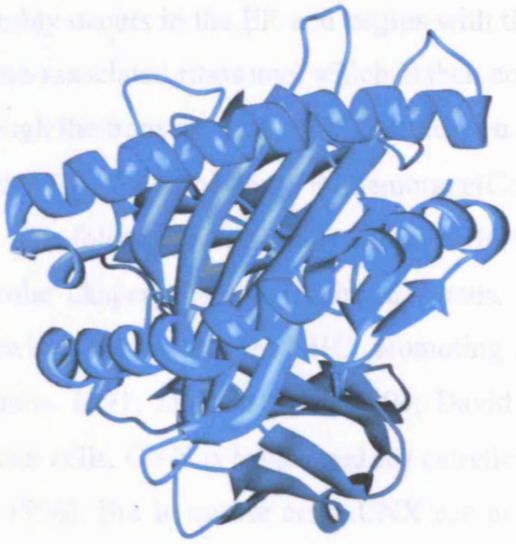


Fig. 1.1: HLA-A2 diagram top view

Showing the antigen-binding cleft, 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

1.2 MHC class I antigen processing and presentation

MHC class I antigen processing starts in the cytosol, where peptides are generated by proteasomes(Cascio et al., 2001; Kloetzel, 2001) and/or other proteases(Reits et al., 2004; Saric et al., 2004; Stoltze et al., 2000) from endogenously synthesized proteins (Fig 1.2). Peptides produced are then transported into the lumen of endoplasmic reticulum(ER) via TAP(Attaya et al., 1992) and, if necessary, they are further trimmed by ER-associated aminopeptidases (ERAAP) to 8-10 amino acids(Serwold et al., 2002; Saric et al., 2002;

York et al., 2002), the length that is generally required for association with MHC class I molecules.

The MHC class I assembly occurs in the ER and begins with the translation of the class I HC in the ER membrane-associated ribosome, which is then co-translationally inserted in the ER membrane through the translocon, Sec61p complex—a channel through which the nascent chain enters the ER lumen or the ER membrane(Corsi and Schekman, 1996; Rapoport et al., 1999). The folding and assembly of the MHC class I complex is assisted by a variety of molecular chaperones and folding enzymes. Calnexin (CNX) interacts transiently with the newly synthesized class I HC, promoting folding and assembly with β2m(Degen and Williams, 1991; Jackson et al., 1994; David et al., 1993; Nossner and Parham, 1995). In human cells, CNX is exchanged for calreticulin (CRT) after HC binds β2m(Sadasivan et al., 1996). But in mouse cells, CNX can also associate with HC-β2m heterodimer(van Leeuwen and Kearse, 1996a).

The association of CNX and/or CRT with class I HC recruits the folding enzyme, ERp57, which then promotes the formation of disulfide bonds in the HC(Molinari and Helenius, 1999; High et al., 2000; Zapun et al., 1998; Oliver et al., 1997). The HC-β2m heterodimer is conformational unstable(Bouvier and Wiley, 1998) and requires the binding of antigenic peptide to complete the maturation process(Paulsson et al., 2001). Efficient peptide loading of MHC class I molecules requires peptide-loading complex (PLC), which includes CRT, ERp57, TAP and tapasin (Cresswell et al., 1999; Cresswell, 2000; Momburg and Tan, 2002; Meunier et al., 2002). The interactions among these molecules stabilize HC-β2m heterodimers and retain them in the ER until the binding of an antigenic peptide completes the assembly process. Upon peptide binding, MHC class I complexes are released from the PLC(Ortmann et al., 1994; Suh et al., 1994; Li et al., 1999) and traffic via the Golgi apparatus to the cell surface(Germain, 1994), where they are surveyed by $CD8^+$ T cells (Fig 1.2). This MHC class I antigen processing and presentation pathway has been regarded as the 'classical' or 'conventional' endogenous class I pathway. However, recent progress in the understanding of the mechanisms of

antigen presentation and activation of T-cell responses suggests that there are other pathways for loading epitopes onto MHC class I molecules(Rock and Shen, 2005), which will be mentioned when appropriate.

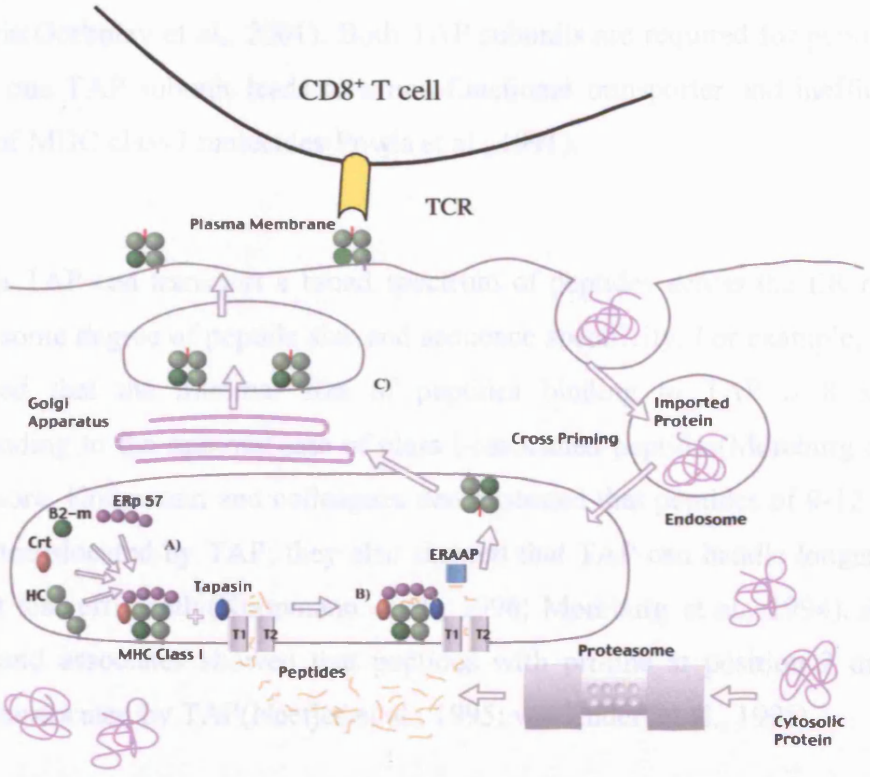
Expression of self peptide/MHC complexes in the thymus is crucial for T cell positive and negative selection, which is important for the development of self-tolerance(Goldrath and Bevan, 1999). Furthermore, MHC class I antigen processing and presentation of foreign proteins at the cell surface for inspection by $CD8^+$ T cells is critical for immune system to eliminate the pathogens(Germain, 1994). Cell surface abundance of MHC class I complex is related to many factors, such as the availability of peptides produced and provided, class I assembly and its subsequent export from the ER, as well as its rate of removal from the cell surface(Elliott and Williams, 2005). However the factors affecting the rate of removal of class I molecules are poorly understood, other factors relating to MHC class I antigen processing and presentation will be discussed in detail in the following sections.

Fig. 1.2 –Class I antigen processing and presentation pathway.

A) HC disulfide bridges form and association with β_2m occurs allowing 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.*



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

1.2.1 Components of MHC class I assembly machinery

1.2.1.1 TAP

TAP belongs to the family of ATP-binding cassette transporters that translocate a broad spectrum of solutes across the ER membrane in an ATP-dependent manner(Ortmann et al., 1994; Suh et al., 1994; Attaya et al., 1992). The TAP complex located in the ER membrane is a heterodimer comprised of TAP1 and TAP2 whose genes are located in the MHC class II region of chromosome 6(Kelly et al., 1992; Spies et al., 1992). Both subunits can be divided into a transmembrane domain (TMD) that binds peptides and forms the translocation pore within the ER membrane, and a cytosolic nucleotide-binding domain (NBD), which energizes peptide translocation across the ER membrane by ATP hydrolysis(Gorbulev et al., 2001). Both TAP subunits are required for peptide transport. Lack of one TAP subunit leads to a non-functional transporter and inefficient peptide loading of MHC class I molecules(Powis et al., 1991).

Although TAP can transport a broad spectrum of peptides across the ER membrane, it exhibits some degree of peptide size and sequence specificity. For example, Momburg et al showed that the minimal size of peptides binding to TAP is 8 amino acids, corresponding to the minimal size of class I-associated peptides(Momburg et al., 1994). Furthermore, Koopmann and colleagues demonstrated that peptides of 9-12 amino acids are best translocated by TAP; they also showed that TAP can handle longer peptides as well, but less efficiently(Koopmann et al., 1996; Momburg et al., 1994). Additionally, Neefjes and associates showed that peptides with proline at position 2 or 3 are very poorly translocated by TAP(Neefjes et al., 1995; van Endert et al., 1995).

In addition to its role as a peptide transporter, TAP has been proposed as a docking station for assembly of class I molecules at the center of the PLC. In support of this hypothesis, Koch et al showed that TAP plays a role in recruiting the adapter protein tapasin by its N-terminal transmembranne segments(Koch et al., 2005; Koch et al., 2004). Moreover, Ortmann et al found that TAP associates with HC-β2m heterodimer to retain it

in the ER(Ortmann et al., 1994). Conversely, in TAP deficient cells, most class I molecules fail to assemble with stably bound peptide due to the shortage of peptide supply and are retained in the ER by tapasin(Grandea, III et al., 2000; Schoenhals et al., 1999). These empty class I molecules that accumulate in the ER are eventually translocated to the cytoplasm for degradation(Van Kaer et al., 1992; Wiertz et al., 1996). Consequently, TAP deficient cells are unable to present intracellular antigens on the cell surface(Hosken and Bevan, 1990; Spies et al., 1992). However, a small number of empty class I molecules do exit the ER in these TAP deficient cells and can be stabilized at the cell surface by addition of class I binding peptides to the culture medium(Townsend and Bodmer, 1989; Cerundolo et al., 1990; Ljunggren et al., 1990; Van Kaer et al., 1992). Moreover, when peptide is delivered directly to the ER in a signal-dependent way, class I molecules of TAP deficient cells become loaded with peptide, dissociate from tapasin in the normal way and traffic to the cell surface(Paulsson et al., 2001). Additionally, these peptide-receptive MHC class I molecules in the absence of TAP can be expressed at the same level as TAP competent cells when they are cultured at 26°C, as the cell surface empty class I molecules become stable at 26°C, whereas they are thermolabile and are degraded at physiological temperature(Ljunggren et al., 1990; De Silva et al., 1999).

Nevertheless, in the absence of TAP, few MHC class I alleles (HLA-A2 and HLA-E molecules) have been showed to bind peptides, which have been proposed to represent signal sequence fragments of secreted/membrane proteins that are cleaved by signal peptidases and loaded in the ER(Henderson et al., 1992; Wei and Cresswell, 1992; Huczko et al., 1993). Another source of TAP-independent peptides presented by class I molecules in TAP deficient cells is the degradation of particular antigen by endolysosomal proteases in antigen presenting cells (APCs). The resulting peptides then bind to MHC class I molecules recycling through, or delivered to the endocytic compartment rather than in the ER, which is regarded as cross-presentation(Shen et al., 2004; Jondal et al., 1996; Ackerman and Cresswell, 2004).

1.2.1.2 Tapasin

Tapasin has been characterized as a member of immunoglobulin superfamily(Ortmann et al., 1997). It is a 48 kDa type I membrane glycoprotein encoded by a gene located within the MHC class II region of chromosome 6 in humans. Structurally, it is comprised of a large N terminal region localized in the lumen of the ER, a single transmembrane domain, and a short C-terminal cytosolic tail, which bears an ER-retention motif(Ortmann et al., 1994; Li et al., 1997; Jackson et al., 1990).

The specific function of tapasin for MHC class I processing is striking. Unlike other chaperones in the ER, such as CNX, CRT, and ERp57, which participate in the general quality control of newly synthesized glycoproteins(Zhang et al., 1997; Peterson et al., 1995; Wada et al., 1995), MHC class I is the only known target molecule for tapasin. Tapasin has been proposed to have several functions in the assembly of MHC class I. (1) It forms a bridge between class I molecules and TAP(Sadasivan et al., 1996). The C-terminal site of tapasin binds to TAP(Momburg and Tan, 2002) and the N-terminus of tapasin(Bangia et al., 1999; Momburg and Tan, 2002) interacts with the α2 domain of human leukocyte antigen-A2 (HLA-A2) [best defined by a mutation in residue 134 of HLA-A2](Peace-Brewer et al., 1996; Lewis et al., 1996) and the α3 domain of the class I HC(Suh et al., 1999; Kulig et al., 1998); (2) It influences the efficiency of peptide binding to TAP in the cytosol(Li et al., 2000), stabilizes TAP, and enhances its ability to transport peptides(Garbi et al., 2003; Lehner et al., 1998); (3) It functions to retain class I molecules in the ER to facilitate peptide loading and optimize the quality of the peptide repertoire(Zarling et al., 2003; Schoenhals et al., 1999; Barnden et al., 2000).

The requirement of tapasin for antigen presentation is well documented(Wright et al., 2004; Momburg and Tan, 2002; Ortmann et al., 1997; Grandea, III and Van Kaer, 2001; Grandea, III et al., 2000). In human and murine tapasin deficient cells, both CRT and ERp57 exhibit reduced association with class I heterodimers(Grandea, III et al., 2000), and the interaction between MHC class I and TAP is impaired(Sadasivan et al., 1996). Furthermore, both TAP expression level and TAP peptide transport are reduced(Garbi et

al., 2003; Grandea, III et al., 1995; Greenwood et al., 1994). As a consequence, MHC class I molecules in tapasin deficient cells are more rapidly transported but at the cost of optimal folding. These empty or sub-optimally loaded MHC class I molecules that reach the surface of tapasin deficient cells rapidly fall apart and the ill-conformed chains are then internalized and degraded. Accordingly, MHC class I surface expression is reduced by 80-90%. However, cell surface expression of MHC class I in human tapasin knockout cell lines is allele specific(Greenwood et al., 1994). For instance, the HLA-B44 allele is highly tapasin dependent, and surface expression of this allele is almost completely lost in tapasin deficient LCL721.220 cells, whereas HLA-B27 allele is partially dependent on tapasin, and seems to be normally expressed at the surface of cells without tapasin. The classical murine MHC class I molecules investigated so far depend on tapasin for surface expression and stability(Peh et al., 1998).

Cell surface peptide receptive class I on tapasin deficient cells can be stabilized by addition of class I binding peptides to the culture medium(Williams et al., 2002b; Ljunggren et al., 1990). Moreover, the antigen presentation defect in tapasin deficient cells can be rescued by reintroduction of tapasin by transfection(Ortmann et al., 1997; Lehner et al., 1998; Bangia et al., 1999). Finally, studies have showed that tapasin-negative mice had reduced selection of $CD8^+$ T cells and impaired CTL viral responses, which again demonstrated that tapasin is critical for peptide-loading and peptide selection(Garbi et al., 2000; Grandea, III et al., 2000).

The requirement for tapasin in the assembly of class I molecules seems due to a decreased TAP expression and peptide translocation into the ER. Evidence for this comes from the work done by Garbi and colleagues who showed that targeting some epitopes into the ER of tapasin$^{-/-}$ cells by a signal sequence rescues the surface class I expression and cytolytic activity by CTLs to the same extent as in wild-type cells, while expression of these epitopes targeted into the cytosol is reduced in the absence of tapasin(Garbi et al., 2003). By contrast, other studies found that a mutant tapasin (N-terminal deletions) that binds TAP but not MHC class I restores peptide supply but fails to restore surface

class I expression(Bangia et al., 1999; Peh et al., 2000). It was argued that tapasin may have functions beyond enrichment of peptides supplier through TAP. This notion is supported by the work of Lehner et al, who demonstrated that mutant tapasin engineered to bind class I not TAP (C-terminal deletions) fails to restore peptide supply to the ER but fully restores the cell surface expression of the tapasin dependent alleles HLA-B8 and HLA-B4402 (Lehner et al., 1998).

Tapasin, as discussed above, is required for the interactions of different components within the PLC. Bangia et al used TAP or β2m deficient cells to study how these interactions within the PLC affect tapasin stability. They found that tapasin is stable in the absence of either TAP or MHC class I interaction. They proposed that tapasin stability may be aided by multiple interactions, any one of which may be sufficient. Furthermore, they demonstrated that, in the absence of TAP, tapasin and class I molecules interact in a prolonged and stable manner(Bangia and Cresswell, 2005), which are consistent with reports that tapasin retains MHC class I HCs in the ER of invertebrate cells(Schoenhals et al., 1999). Thus, although class I molecules in TAP deficient cells do not bind peptide, they are more stable compared with cells lacking tapasin(Grandea, III et al., 1997).

1.2.1.3 ERp57

ERp57 is a member of the protein disulfide isomerase (PDI) family whose function includes disulfide bond isomerisation, reduction and oxidation(Lindquist et al., 2001; Ferrari and Soling, 1999). ERp57 has been identified as a component of the MHC class I peptide-loading complex, and it interacts with other members of PLC(Hughes and Cresswell, 1998; Morrice and Powis, 1998). For instance, ERp57 and tapasin are connected by a disulfide bond between Cys 95 of tapasin and Cys57 of ERp57, and this interaction is independent of CRT and CNX, or availability of monoglucosylated N-linked glycans(Peaper et al., 2005; Dick et al., 2002). Garbi et al have suggested that the interaction of ERp57 with tapasin may induce a conformational change in the N-terminus

of tapasin, which enables the latter to bind MHC class I molecules and bridges them to TAP(Garbi et al., 2006). Furthermore, ERp57 has been demonstrated to associate with the tip of an arm-like P-domain of CRT and CNX(Leach et al., 2002; Frickel et al., 2002; Pollock et al., 2004). Both CRT and CNX interact with class I HC via their N-linked glycans(Hammond et al., 1994; Peterson et al., 1995; Wada et al., 1995; Ware et al., 1995). Therefore, ERp57 has been proposed to function as a 'co-chaperone' of CNX and CRT. It forms one to one complexes with both CNX and CRT to modulate glycoprotein folding(Oliver et al., 1997; Oliver et al., 1999; Elliott et al., 1997; van der Wal et al., 1998; Molinari and Helenius, 1999). In this way ERp57 facilitates disulfide-bond isomerization of class I molecules by transiently forming mixed disulfides with them(Farmery et al., 2000; Molinari and Helenius, 1999; High et al., 2000; Ellgaard et al., 2001; Ellgaard and Helenius, 2001). In agreement with this, Elliott and Zapun have observed that the disulfide isomerase activity of ERp57 is greatly enhanced in the presence of CNX or CRT, as they are brought into close proximity by their respective interaction with the lectin, their relative local concentration is increased(Elliott et al., 1997; Zapun et al., 1998). There are two disulfide bonds formed in the class I HC, the first one in the α3 domain and the second one in the α2 domain. The importance of correctly formed disulfide bonds is demonstrated by mutation analysis in either of these bonds, which leads to misfolding of class I in the ER and results in reduced cell surface expression(Miyazaki et al., 1986; Warburton et al., 1994).

Nevertheless, the importance of ERp57 in class I HC oxidation has been called into question by the finding that class I HC of B cells lacking ERp57, derived from ERp57$^{-/-}$ mice generated by a targeted ERp57 knockout, displays a normal redox status, indicating that ERp57 may not play a unique role in HC oxidation(Garbi et al., 2006). In addition to this surprising finding, they demonstrated that, in ERp57-deficient cells, although the interaction of tapasin with TAP is not affected, the association of class I molecules to the TAP complex is reduced by about 90-95%; very few class I molecules are present in the loading complex; and the surface class I expression and stability are reduced. They concluded that ERp57 is central for recruitment of MHC class I molecules into the loading complex and it is an essential component required for stable assembly of the

peptide-loading complex. The molecular mechanism for ERp57-mediated binding of class I molecules by tapasin and recruitment into the PLC is not yet understood.

Apart from the controversial role ERp57 plays in HC oxidation, Powis et al have showed that ERp57 can act to reduce partially folded MHC class I molecules that are produced due to lack of high-affinity peptides. Reduction of class I molecules leads to unfolding and targeting for degradation, which is important for quality control in the ER. They suggest that ERp57 is likely to play a dual role in MHC class assembly, being directly involved in the formation and/or breakage of disulfide bonds in class I HCs(Antoniou et al., 2002).

1.2.1.4 Calnexin

Calnexin (CNX) is a 65 kDa type I membrane protein and has an ER localization sequence at its C-terminus(Ou et al., 1993). Like its luminal homologue CRT, CNX also possesses a proline rich central P domain, which has been shown to bind to ERp57(Leach et al., 2002; Frickel et al., 2002). This central P domain has also been identified to be the lectin site of calnexin capable of binding oligosaccharide(Vassilakos et al., 1998).

CNX is regarded as a general chaperone that assists in the folding of newly synthesized glycoproteins in the ER, which contributes to ER quality control. MHC class I is one of CNX substrates. It has been demonstrated that CNX interacts with MHC class I HC upon its co-translational insertion in the ER membrane to facilitate the folding of HC and stabilize it from aggregation as well as promote its assembly with β2m(David et al., 1993; Degen and Williams, 1991; Degen et al., 1992; Hochstenbach et al., 1992). In mouse cells, CNX associates with both free class I HCs and class I-β2m dimmers(Degen et al., 1992). In human cells, however, it predominantly associates with free class I HCs(Ortmann et al., 1994; Sugita and Brenner, 1994; Nossner and Parham, 1995). This maybe due to one fact that human MHC class I molecules possess a single glycosylation site at position 86(Parham et al., 1977); while in addition to position 86, mouse HCs are

also glycosylated at Asn176 for K^b and Asn176/Asn256 for D^b (Evans et al., 1982; Maloy and Coligan, 1982; Nathenson et al., 1981; Helenius and Aebi, 2001). Support for this hypothesis comes from studies of mutant human HC with a second glycan introduced which revealed an assembly process similar to mouse HC with respect to lectin association(Zhang and Salter, 1998).

The requirement for CNX to associate with class I HC is not very clear since the assembly, transport, and the cell surface expression of MHC class I molecules is unaffected in CNX deficient cells(Sadasivan et al., 1995; Scott and Dawson, 1995; Prasad et al., 1998). It seems likely that there is redundancy in the ER and that other chaperones can perform the same function as CNX in its absence. Immunoglobulin binding protein (BiP) is a possible candidate as BiP has been shown to associate with free human class I HC alleles in $\beta 2m^{-/-}$ cells (but not with mouse alleles)(Paulsson et al., 2001; Nossner and Parham, 1995). Nevertheless, it is still believed that the interaction between CNX and HC could serve to increase the efficiency of HC folding and may protect nascent chains from immediate degradation(David et al., 1993; Degen and Williams, 1991; Degen et al., 1992; Nossner and Parham, 1995; Hochstenbach et al., 1992).

1.2.1.5 Calreticulin

CRT is a ubiquitous protein, found in a wide range of species. It is highly conserved, with over 90% amino acid identity existing between human, rabbit, and mouse forms of protein. CRT was first isolated by Ostwald and MacLennan in 1974(Ostwald and MacLennan, 1974), followed by molecular cloning of the protein in 1989 by Smith and Kock, and Fliegel(Smith and Koch, 1989; Fliegel et al., 1989a). It appears most abundant in the endoplasmic reticulum (ER)(Nash et al., 1994; Sonnichsen et al., 1994), but has also been detected in other cellular compartments, such as the nucleus(Opas et al., 1991), cytoplasm and cell surface(Michalak et al., 1992; Arosa et al., 1999; Wiest et al., 1997; Xiao et al., 1999) and it may even be secreted in the bloodstream(Eggleton and Llewellyn, 1999).

1.2.1.5.1 The CRT gene and protein

The CRT gene consists of 9 exons and 8 introns and spans approx. 3.6 kb or 4.6 kb of human or mouse genomic DNA respectively(Waser et al., 1997). The genes of human and mouse have been localized to chromosomes 19 and 8 respectively(Rooke et al., 1997). The nucleotide sequences of the human and mouse genes show greater than 70% identity. Surprisingly, only one mRNA species encoding CRT was identified in a variety of different tissues, and there is no evidence for RNA splicing, indicating a high level of conservation of the gene. The promoter of the human and mouse CRT genes contain several putative regulatory sites and is activated by Zn2+ and heat shock(Nguyen et al., 1996; Conway et al., 1995). Expression of CRT is also induced by viral infection(Zhu, 1996), by amino acid deprivation(Plakidou-Dymock and McGivan, 1994) and in stimulated cytotoxic T-cells(Burns et al., 1992).

CRT protein contains 400 amino acids and has a calculated molecular mass of 46 kDa. Anomalous migration in SDS-PAGE, likely due to its highly negative charge (PI=4.7) and/or other structural features, results in CRT's appearance as an appropriate 60kDa protein(Fliegel et al., 1989a). Moreover, CRT can exist as a 120 kDa dimer(Rizvi et al., 2004). As an ER resident protein, CRT has an N-terminal cleavable 17 amino acid signal sequence to target the protein to the ER and a C-terminal KDEL ER retrieval signal for the retention of CRT in the ER(Fliegel et al., 1989a; Munro and Pelham, 1987). Structurally and functionally, CRT can be divided into three domains, N-domain, P domain and C-domain(Smith and Koch, 1989; Nakamura et al., 2001b), which is supported by the NMR structure of the central, proline-rich P-domain of CRT revealing highly unusual arrangements(Ellgaard et al., 2001) (Fig 1.3).

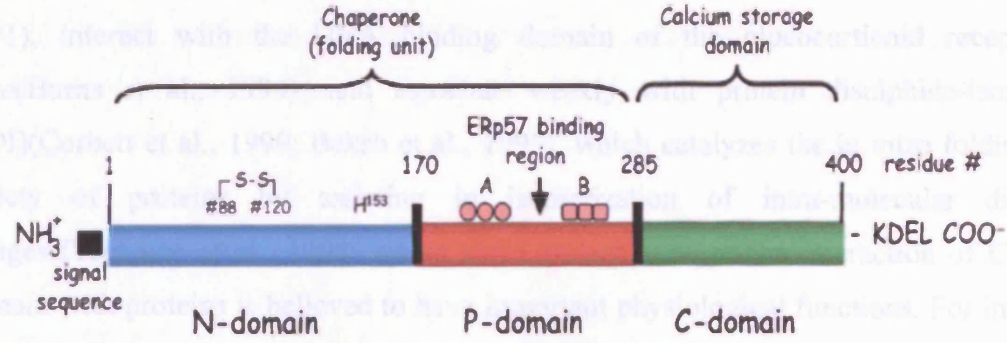


Fig. 1.3 Schematic structure of CRT

Adapted from - The International Journal of Biochemistry and Cell Biology. 2005, 37: 260-266; P. Gelebart et al. Calreticulin, a Ca^{2+} -binding chaperon of the endoplasmic reticulum

The N-domain of CRT (residues 1-180) is coded by exons 1, 2, 3 and 4, and is the most conserved domain among CRTs from different species(Michalak et al., 1992; Krause and Michalak, 1997). Based on the X-ray structure of soluble calnexin(Schrag et al., 2001) and the NMR structure determined for the P-domain of CRT(Ellgaard et al., 2001), the N-domain of CRT is predicted to form a highly folded globular structure (Fig 1.4). This domain has three cysteine residues, and the location of these amino acid residues is conserved in CRT from high plants to that in humans. Two out of three cysteine residues form a disulphide bridge (Cys120-Cys146), which may be important for proper folding of the N-domain of CRT(Michalak et al., 1992). Furthermore, the N-domain of CRT contains 5 histidine residues, which are involved in zinc binding to the protein(Baksh et al., 1995). This domain contains a high capacity, low affinity zinc binding site(Khanna et al., 1986). The importance of 5 histidine residue at position 153 (His153) has been demonstrated by the finding that mutation of His153 in CRT's N domain destroys the protein's chaperone function, and biophysical analysis of the His153 mutant revealed that

a conformational change in the CRT mutant may be responsible for the loss of it's chaperone function(Guo et al., 2003). Additionally, it has been reported that the N domain of CRT can bind the cytoplasmic domains of integrin α subunits(Rojiani et al., 1991), interact with the DNA binding domain of the glucocorticoid receptor in vitro(Burns et al., 1994), and associate weakly with protein disulphide-isomerase (PDI)(Corbett et al., 1999; Baksh et al., 1995), which catalyzes the in vitro folding of a variety of proteins by assisting in isomerization of intra-molecular disulfide bridges(Freedman et al., 1994; Noiva and Lennarz, 1992). The interaction of CRT N-domain with proteins is believed to have important physiological functions. For instance, interaction of the CRT N–domain with PDI inhibits PDI chaperone function as measured by the ability of PDI to refold scrambled RNase A(Baksh et al., 1995). Studies revealed that these protein-protein interactions are regulated by calcium binding to the C-domain of CRT(Corbett et al., 1999). Finally, the N-domain of CRT has been shown to inhibit proliferation of endothelial cells and suppresses angiogenesis(Pike et al., 1998).

The P-domain of CRT (residues 181-280) may be encoded by exons 5, 6 and 7(Michalak et al., 1992; Krause and Michalak, 1997) and forms an extended-arm structure (Fig 1.4)(Ellgaard et al., 2001). It is rich in proline(Fliegel et al., 1989a) and contains two sets of three sequences repeats (Repeat A: amino acid sequence PXXIXDPDAXKPEDWDE, and repeat B: amino acid sequence GXWXPPXIXNPXYX), which is unique to CRT and its homologue calnexin(Watanabe et al., 1994; Bergeron et al., 1994). The sequence of the P domain is highly conserved from plants and parasites to mammals. For example, there is only one amino acid difference between the p-domain of human and mouse forms of CRT (see appendix). Functionally, like the N-domain of CRT, P-domain of CRT is also involved in protein-protein interactions. Leach et al reported that the P domain of CRT interacts with ERp57 through the tip of the arm domain(Leach et al., 2002). This is supported by the work from Ellgaard and colleagues, who showed the nuclear magnetic resonance (NMR) structure for the P-domain of CRT(Ellgaard et al., 2001). Furthermore, the P-domain of CRT also interacts strongly with PDI(Baksh et al., 1995) and perforin(Andrin et al., 1998; Fraser et al., 1998). These protein-protein interactions may lead to modulation of their functions. For example, in the presence of PDI, CRT does not

bind Ca^{2+} with high affinity(Baksh et al., 1995). In general, the P-domain of CRT, together with the N-domain of CRT, has been proposed to form a functional "protein-folding module" (Michalak et al., 1992). Moreover, like the P-domain of calnexin, the P domain of CRT has also been identified to be the lectin site of CRT capable of binding oligosaccharide(Vassilakos et al., 1998). The P-domain also plays a role in Ca^{2+} storage in the lumen of ER. It binds Ca^{2+} with a high affinity, low capacity(Baksh and Michalak, 1991).

The C-domain of CRT (residues 281-400) is encoded by exons 8 and 9(Fliegel et al., 1989b). This domain is highly acidic (37 of its final 57 residues are aspartic or glutamic acid) and terminates with the KDEL ER retrieval signal(Fliegel et al., 1989b; Smith and Koch, 1989). The C domain binds Ca^{2+} with high capacity but low affinity(Fliegel et al., 1989b) (Fig 1.4), which enables CRT to be one of the major proteins regulating the process of Ca^{2+} homeostasis in the ER. However, CRT determines the Ca^{2+} storage capacity, not the free Ca^{2+} concentration of the ER(Nakamura et al., 2001b). Moreover, studies have indicated that Ca^{2+} binding to this domain of CRT plays a regulatory role in the control of CRT interaction with other proteins or chaperones, such as PDI and ERp57(Corbett et al., 1999). CRT associates with these ER proteins in a Ca^{2+}-dependent manner to modulate their functions(Corbett et al., 1999; Booth and Koch, 1989; Lodish and Kong, 1990; Corbett et al., 2000). For example, CRT interacts with PDI only when the Ca^{2+}concentration is below 100 μM, a Ca^{2+} concentration found upon emptying of the Ca^{2+} stores, but the protein complex dissociates upon increased Ca^{2+} concentration (>500μM). In addition, although the formation of the CRT-ERp57 complex is initiated by a Ca^{2+} independent conformational change in ERp57, it is followed by additional Ca^{2+} dependent conformational changes in the complex(Corbett et al., 1999). Therefore, it seems that many functions proposed for CRT could be affected by its effects on the ER Ca^{2+} storage capacity(Nakamura et al., 2001b; Bastianutto et al., 1995).

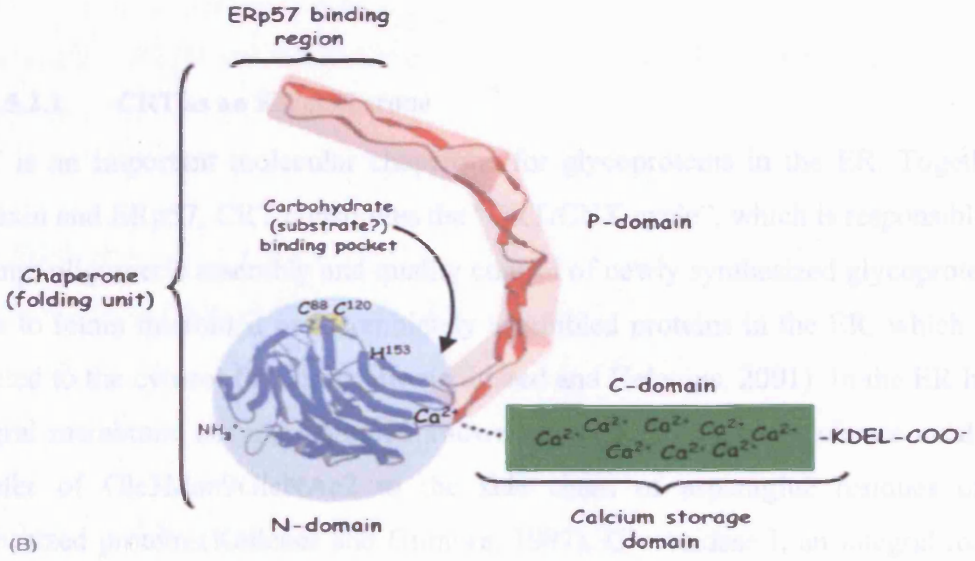


Fig. 1.4: 3D structure of CRT

Adapted from - The International Journal of Biochemistry and Cell Biology. 2005, 37: 260-266; P. Gelebart et al. Calreticulin, a Ca^{2+} -binding chaperon of the endoplasmic reticulum

1.2.1.5.2 CRT function

Although CRT was first identified as a Ca^{2+} binding protein(Ostwald and MacLennan, 1974), later multiple functions have been postulated for CRT, including functions of CRT within the ER (chaperone(Molinari et al., 2004) as well as Ca^{2+} storage, signaling(Nakamura et al., 2001b; Arnaudeau et al., 2002)) and extra-ER functions (CRT-dependent modulation of cell adhesion(Opas et al., 1996; Leung-Hagesteijn et al., 1994; Dedhar, 1994)). Moreover, CRT plays a crucial role during early cardiac development(Michalak et al., 2004; Nakamura et al., 2001a). In addition to its physiological function, CRT has been implicated in a number of pathological processes, such as autoimmune diseases(Eggleton and Llewellyn, 1999; Karska et al., 1995) and

cancer (Kageyama et al., 2004; Hong et al., 2004). In the following chapters, however, only the functions of CRT that relate to this study will be discussed.

1.2.1.5.2.1 CRT as an ER chaperone

CRT is an important molecular chaperone for glycoproteins in the ER. Together with calnexin and ERp57, CRT constitutes the "CRT/CNX cycle", which is responsible for the folding, oligomeric assembly and quality control of newly synthesized glycoproteins, and helps to retain misfolded or incompletely assembled proteins in the ER, which are then targeted to the cytosol for degradation(Ellgaard and Helenius, 2001). In the ER lumen an integral membrane enzyme complex known as oligosaccharide transferase catalyses the transfer of Glc3Man9GlcNAc2 to the side chain of asparagine residues of newly synthesized proteins(Kelleher and Gilmore, 1997). Glucosidase I, an integral membrane protein, then removes the terminal α1,2-linked glucose (Fig 1.5), followed by elimination of one of the two α1,3-linked glucose by Glucosidase II, a soluble luminal protein, to produce the Glc1Man9GlcNAc2 processing intermediate. This is the specific ligand of CRT and CNX(Hammond and Helenius, 1994; Ware et al., 1995). The second α1,3-linked glucose exposed by a dynamic association of glycoprotein with CNX or CRT is trimmed by Glucosidase II, which is important in the regulation of glycoprotein-chaperone interactions(Trombetta, 2003; Helenius and Aebi, 2004). If the glycoprotein is folded correctly, it escapes from the folding cycle. However, If the released de-glucosylated glycoprotein does not acquire its native state, the terminal α1,3-linked glucose is once again added back to the oligosaccharides to generate monoglucosylated glycans by the UDP-glucose: glycoprotein glycosyltransferase which serves as a folding sensor recognizing and re-glucosylating non-native glycoproteins(Sousa and Parodi, 1995). This de-glucosylation/glucosylation cycle may be repeated several times before a newly synthesized glycoprotein is properly folded. The misfolded proteins are hydrolyzed by ER α-mannosidase I to target them for retrotranslocation through Sec61 channel to be degraded by the proteasome(Cabral et al., 2001).

CRT binding to monoglucosylated carbohydrate is sensitive to changes in Ca^{2+} concentrations, suggesting the changes in the ER luminal Ca^{2+} concentrations may have profound effects on the formation of the chaperon-substrate complexes, which subsequently affects its ability to assist in the folding of glycoproteins(Corbett et al., 2000; Vassilakos et al., 1998).

The importance of CRT for glycoprotein folding comes from the work by Molinari and colleagues, who showed that CRT depletion specifically accelerates the maturation of cellular and viral glyoproteins with a modest decrease in folding efficiency(Molinari et al., 2004). Additionally, the chaperone function of CRT has been demonstrated in connection with the folding of insulin receptor monomers(Bass et al., 1998), and the assembly of nascent T-cell receptor proteins(van Leeuwen and Kearse, 1996b).

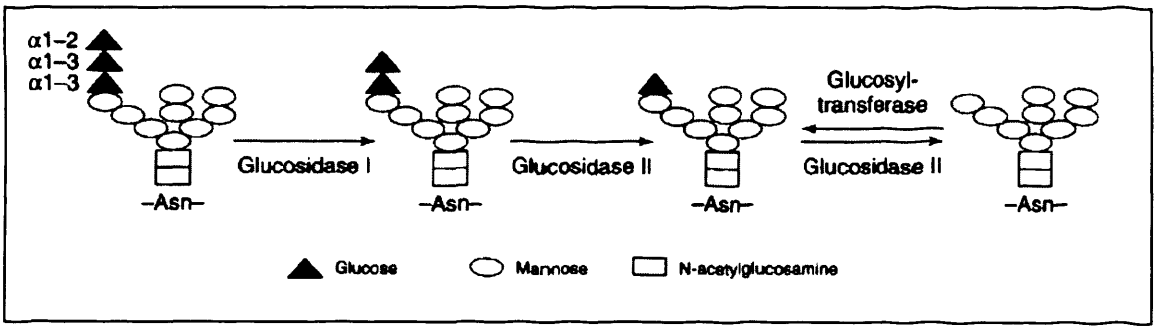


Fig. 1.5: Glucose modification by enzymes in the ER

Adapted from – Trends in cell biology, 1997, 17:193. Ari Helenius et al. Calnexin, calreticulin and the folding of glycoproteins

Although it is generally accepted that CRT is an important chaperone, there is debate about whether CRT binds only to carbohydrate or if it also binds directly to polypeptides. Two models have been proposed to describe the interactions of CRT with newly synthesized proteins. In the lectin-only model, CRT functions as lectin binding to glycoproteins exclusively through lectin-oligosaccharide(Peterson et al., 1995). Evidence

for this model is that CRT-glycoprotein complexes are dissociated by the action of glucosidase II, which removes the single glucose residue, and that glucosidase inhibitors, such as castanospermine, block CRT-glycoproteins complex formation(Zhang et al., 1997; Peterson et al., 1995; Hammond and Helenius, 1994). Furthermore, CRT was shown to be unable to discriminate between reduced and native forms of monoglucosylated RnaseB, and the complex between CRT and RnaseB could only be dissociated in vitro by digestion with glucosidase II(Rodan et al., 1996). Finally, the in vitro study carried out by Wearsch and colleagues showed that CRT bound as efficiently to peptide-loaded MHC class I molecules as it did to folding intermediates, namely free class I HC and empty HC-β2m heterdimers(Wearsch et al., 2004). The primary lectin site of CRT, demonstrated by Leach and colleagues, resides within the N-domain with a weaker secondary site within the P-domain(Leach et al., 2002). In the second model known as the dual-binding model, CRT is proposed to bind to unfolded glycoprotein through interactions with both carbohydrate and exposed hydrophobic polypeptide sequences. Therefore, the dissociation of CRT-glycoprotein complexes occurs through the action of glucosidase II as well as a conformational change in the polypeptide-binding site. Support for this model comes from the finding that CRT binds to several non-glycosylated proteins including ERp57(Corbett et al., 1999) and PDI(Baksh et al., 1995). Moreover, CRT has been shown to discriminate native and non-native conformations of non-glycosylated proteins in vitro(Svaerke and Houen, 1998; Saito et al., 1999), and Jørgensen et al have showed that CRT interacted better with unfolded ovalbumin than with native ovalbumin(Jorgensen et al., 2000). Additionally, a study carried out by Leach et al showed that CRT suppressed aggregation of unfolded proteins via a polypeptide binding site, which is located within the N-domain of CRT, but requires the P-domain for its full chaperone function(Leach et al., 2002). However, it has not yet been possible to resolve this debate with the current available experimental substrates and methods.

As mentioned above, CRT and CNX recognize identical glycan moieties(Vassilakos et al., 1998), but, the substrate glycoproteins with which the two proteins interact are not identical(Danilczyk et al., 2000). What determines whether unfolded glycoproteins bind to CRT or CNX? The answer may be dependent on the number and position of N-linked

oligosaccharide chains(Molinari and Helenius, 2000). Extensive mutagenesis studies of the glycosylation sites in influenza hemagglutinin revealed that CRT binds preferentially to N-glycans located at the membrane-distal domain of the molecule, whereas CNX binds equally well to the membrane-distal and membrane-proximal N-glycans(Hebert et al., 1997). It was also suggested the proteins that fold in the ER make use of proteins in different combinations and different orders(Molinari and Helenius, 2000). In some cases CRT and CNX bind to the same glycoprotein, but they bind different oligosaccharadides. For example, Harris et al showed that of the three glycosylation sites in the mouse L^d HC, removal of the site in the membrane distal α1 domain (residue 86) ablates CRT binding, whereas CNX binding is unaffacted(Hebert et al., 1997; Harris et al., 1998). Alternately CRT and CNX may bind to the same glycoprotein at different stages of maturation process(van Leeuwen and Kearse, 1996a). For some substrates, the difference may be simply due to the topological environment-that is CRT being soluble, whereas CNX being membrane bound. This is supported by evidence that the pattern of bound proteins to CNX was similar to that of CRT when the ectodomain of CNX was expressed as a soluble ER protein(Danilczyk et al., 2000). However there are examples where the ectodomain of CNX cannot replace CRT. For example, Gao and colleagues have shown that transfecting CRT deficient cells with CRT cDNA but not with soluble calnexin cDNA restored cell surface MHC class I expression(Gao et al., 2002). Furthermore, the spectrum of proteins recognized by the membrane-anchored CRT was remarkably similar to that observed with CNX(Danilczyk et al., 2000; Wada et al., 1995). Therefore, a luminal versus membrane topology also appears to play an important role in determining the differential binding specificities of CRT and CNX.

1.2.1.5.2.2 CRT and MHC class I mediated antigen processing

Structurally, CRT is a member of the PLC and it interacts with other components of PLC. After HC is assembled with β2m, CRT associates with MHC class I though the glycan on HC(Sadasivan et al., 1996; Solheim et al., 1997), especially the N-linked glycan in the α1 HC is required for the interaction of the HC with CRT(Harris et al., 1998; Turnquist et al., 2002). The association of CRT with MHC class I molecule is not dependent on either

TAP(Harris et al., 1998) or tapasin(Sadasivan et al., 1996; Solheim et al., 1997). For instance, Turnquist and co-workers demonstrated that in tapasin deficient cells, CRT still can interact with class I(Turnquist et al., 2002). Moreover, class I HC mutants lacking tapasin interaction retained normal association with CRT(Paquet and Williams, 2002). The interaction of CRT with HC appears to be a prerequisite for the association of class I with TAP via tapasin. For example, Elbein and colleagues have showed that class I alleles that associate well with CRT also associate well with TAP(Elbein, 1991). Furthermore, the drug castanospermine, which blocks the action of glucosidase I and II, has been shown to affect HLA class I association with both CRT and TAP, and the authors argued that it is likely that castanospermine inhibits class I-TAP association because it inhibits the prior interaction of CRT with class I-β2m dimers(Elbein, 1991; Sadasivan et al., 1996). Further evidence comes from the work by Harris that mutation of the residue at position 86 alters the Asn-linked glycosylation site and leads to PLC disruption through poor CRT association(Harris et al., 1998; Harris et al., 2001), which is in agreement with another study showing that tapasin does not interact with class I in the absence of CRT interaction with class I(Turnquist et al., 2002). In addition, Gao et al have found that the incorporation of class I molecules into PLC is lower in CRT deficient K42 cells compared with that of CRT competent K41 cells(Gao et al., 2002). Although CRT-class I can be present as an independent complex in the ER, the vast majority of these molecules are simultaneously assembled with three additional accessory proteins: ERp57, tapasin and TAP(Sadasivan et al., 1996; Harris et al., 1998; Solheim et al., 1997). CRT also interacts with ERp57. Evidence for this comes from the biochemical and NMR studies which revealed that the P domain of CRT interacts with ERp57 through several residues located at the tip of its P-domain(Frickel et al., 2002), which is also demonstrated by gel filtration chromatography(Zapun et al., 1998), cross-linking and native gel analysis(Oliver et al., 1999), and by a solid-phase binding assay(Leach et al., 2002). Based on the interactions of CRT with both MHC class I and ERp57, a molecular mechanism describing how CRT in concert with ERp57 facilitates the folding of MHC class I has been proposed. The flexibility of the P domain of CRT may provide a mechanism whereby CRT can effectively optimize interactions between MHC class I and ERp57 to allow the ERp57 to catalyze the formation and rearrangement of two intra-

chain disulfide bonds in MHC class I HC (α2 and α3 domains)(Bouvier, 2003). On the basis of these data, CRT seems to play a central role in the formation of the MHC class I PLC.

Functionally, CRT is critical for the assembly of MHC class I. Studies have found that CRT preferentially binds to open conformations of MHC class I(Sadasivan et al., 1996; Harris et al., 1998) and is not associated with class I after peptide-induced folding(Harris et al., 1998), which suggests that CRT could serve to stabilize and retain the HC-β2m heterodimer in the ER until the antigenic peptide is loaded(Harris et al., 1998; Danilczyk et al., 2000). Furthermore, in vitro studies of the effects of CRT on class I assembly showed that, although in the absence of other ER components of the PLC CRT does not enhance the extent of MHC class I assembly with peptides, CRT does promote the folding of HLA class I molecules(Culina et al., 2004) and inhibit the aggregation of peptide loaded or receptive class I and class I HCs(Wearsch et al., 2004; Mancino et al., 2002). Additionally, the importance of CRT for the quality of MHC class I molecules was highlighted by cells (K42) from CRT deficient mice, which have impaired peptide loading onto class I molecules (the efficiency of peptide loading was reduced by 50-80%), resulting in the accelerated export of peptide-receptive class I molecule from the ER with low cell surface class I expression and low stability of cell surface-expressed class I molecules. Conformation dependent antibodies showed that a greater proportion of MHC class molecules in the ER remain in the peptide receptive state in K42 (CRT-/-) than in K4 1(CRT+/+). Most importantly, 3 of 4 tested epitopes failed to be presented by a calreticulin deficient cell line, implying its critical role in antigen processing for T cell recognition(Gao et al., 2002). Although evidence is accumulating that CRT is critical for efficient MHC class I assembly, the precise role of CRT and the mechanism underlying the essential role in class I assembly remains elusive.

1.2.1.5.2.3 CRT and cell adhesion, cell spreading

CRT has been reported to modulate cell adhesion by intracellular and extracellular mechanisms in several locations, both inside and outside the cell(Michalak et al., 1999;

Johnson et al., 2001). Accordingly, changes in levels of CRT expression affect cell shape, motility, and the cytoskeleton. For example, overexpression of CRT in L fibroblasts increases cell-substratum attachment and establishes epithelial-like cell-cell junctions. Thus, cells look more spread and have more cell projections(Opas et al., 1996) (Fig 1.6), whereas transient down-regulation of CRT reduces attachment of cells to extracellular substrate, which causes cells to be round and smoother than the control cells (Opas et al., 1996; Leung-Hagesteijn et al., 1994). In addition, embryonic stem (ES) cells lacking CRT have impaired cell adhesion(Leung-Hagesteijn et al., 1994). What mechanisms does CRT regulate cell adhesiveness?

CRT might modulate cell adhesion from within the cell through an interaction with integrin tails. The integrins are important mediators of cell adhesion to extracellular ligands. They belong to a family of heterodimeric, transmembrane glycoproteins that are formed by the noncovalent association of α and β subunits(Hynes, 1992; Ruoslahti and Pierschbacher, 1987). On the cell exterior, domains of the α and β subunits form a ligand binding site through which integrins can mediate cell binding to substrates including extracellular matrix molecules (cell-substratum adhesions) and other cell surface proteins (cell-cell adhesion). Once adhered to a substratum, cells recognizing their cytoskeleton convert their shape from sphere to flattened, extended forms. Interior to the plasma membrane, the cytoplasmic domains of integrin subunits can interact with intracellular proteins, such as CRT, calcineurin or calmodulin. It has been suggested that CRT interacts with the KXGFFKR motif in the cytoplasmic domain of the integrin α chain through mainly its N-domain(Michalak et al., 1992). Further studies have demonstrated that CRT deficient ES cells have impaired integrin-mediated adhesion, although integrin expression is unaltered; and expression of recombinant CRT in double CRT knockout ES cells by CRT cDNA transfection rescued integrin-mediated adhesion(Coppolino et al., 1997). But opinion is still divided as to whether CRT dependent integrin mediated cell adhesion is through CRT direct binding to cytoplasmic tails of the integrin or through signaling from within the ER. There is no solid evidence to prove the presence of CRT in the cytosol despite the ability of CRT to bind to integrin tails in vitro(Arvan et al., 1991).

Furthermore, targeting of CRT to the cytoplasm either by microinjection or by expression of leaderless CRT has no effect on cell adhesion (Michalak, M.1996, Careticulin. R. G. Landers, Georgetown. 207 pp). Up to now, the exact mechanism by which CRT modulates integrin function is not very clear.

Alternatively, CRT may influence cell adhesion indirectly from the ER by modulation of gene expression of adhesion-associated molecules, such as vinculin, a cytoskeletal protein essential for cell substratum and cell-cell attachments. Support for this opinion comes from the work done by Opas and colleagues that showed both vinculin protein and mRNA levels are increased in L fibroblasts over-expressing CRT and are down-regulated in cells expressing reduced levels of CRT. By contrast, expression of other adhesion-related proteins, such as actin ,talin, $\alpha 5$ and $\beta 1$ intergrins, pp125 focal adhesion kinase, or α-catenin, is unaltered(Opas et al., 1996). However, the mechanism of CRT effect on vinculin gene activity is currently unknown.

Finally, CRT might affect cell adhesion from the cell surface. CRT has been identified on the surface of a wide variety of cell types, such as human leukocytes(Wiest et al., 1997; Arosa et al., 1999), endothelial cells(Malhotra, 1993) and B16 mouse melanoma cells(Davis et al., 1999). Cell surface CRT was also reported to complex with integrins(Eaton et al., 2000). Furthermore, antibodies against CRT can prevent spreading of B16 cells(Davis et al., 1999). How CRT escapes ER retention and is translocated to the cell surface remains to be answered.

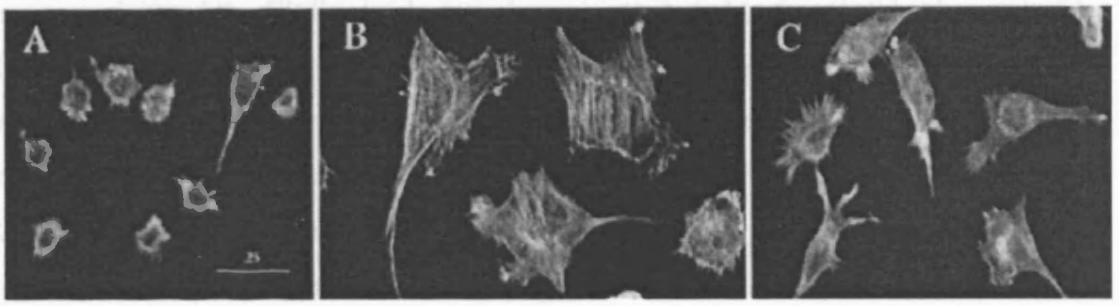


Fig. 1.6 CRT affecting cell adhesion and spreading

(A) Calreticulin underexpressers. (B) Calreticulin overexpressers. (C) Control mock transfectants.

Adapted from – The Journal of Cell Biology, Volume 135, Number 6, Part 2, December 1996 1913-1923. Michal Opas, Calreticulin Modulates Cell Adhesiveness via Regulation of Vinculin Expression

1.2.1.5.2.4 CRT as a chaperone of peptides

It has been proposed that the peptides exist in association with heat shock proteins (Hsps) both in the cytosol and in the ER, and these Hsps are the peptide-binding proteins that transport peptides(Srivastava et al., 1994). One study has shown that free peptides introduced into the cytosol are presented quite inefficiently by class I molecules as compared with peptides chaperoned by Hsps, such as Hsp70 or Hsp90, and the presentation is both peptide-dependent and –specific(Binder et al., 2001). Furthermore, it has been established that ER resident chaperone proteins, such as GRP94, BIP and PDI, display peptide-binding activity(Wearsch and Nicchitta, 1997; Spee and Neefjes, 1997; Noiva et al., 1991; Lammert et al., 1997b).

Because of the established role of CRT in the genesis of class I/peptide complexes and the propensity of ER chaperones to display peptide-binding activity, CRT has been investigated for its role in chaperoning pepides. CRT has been shown to bind to antigenic peptides transported into the ER by TAP in cells(Spee and Neefjes, 1997) and several nonglycosylated peptides in vitro(Basu and Srivastava, 1999; Saito et al., 1999; Jorgensen et al., 2000). Furthermore, in vitro incubation of such CRT-antigenic peptide

complexes with APCs allows cross-presentation of the peptide to CTLs, and the immunogenicity of the CRT-peptide complexes is attributed to the peptide chaperoned by CRT molecule(Basu and Srivastava, 1999; Nair et al., 1999). Additionally, a study by Binder and colleagues showed that CRT associated with peptide in the ER acted as a nonspecific adjuvant present in the cells since they found that peptides chaperoned by CRT or other Hsps are the necessary and sufficient source of antigen transferred to APCs for priming CTL responses(Binder and Srivastava, 2005). Finally, CRT purified from tumour cells has been shown to elicit tumour specific CTL responses in vivo, which has been attributed to the ability of CRT to bind a variety of tumour specific peptide antigens within the cells, and cross present these peptides via class I molecules of APC(Basu and Srivastava, 1999; Nair et al., 1999).

The function of CRT as a peptide chaperone in antigen presentation has also been demonstrated at the DNA level. Cheng and colleagues explored the linkage of CRT to the DNA of a model tumor antigen, human papilloma virus type-16 E7, for the development of a DNA vaccine. They found that the use of CRT chimerically linked to E7 DNA elicited strong E7-specific CTL immune responses and generated a significant CTL-dependent protective effect against E7-expressing tumors(Cheng et al., 2001).

The mechanism by which CRT-peptide complex elicits immunity is however not well understood. One of the possible mechanisms for the chaperone effect of CRT is directly enhancing MHC class I presentation by binding of CRT to peptides to protect them from degradation by ER peptidases, a function demonstrated by other ER peptide chaperons such as gp96, gp170, ERp72, ERp57(Menoret et al., 1999; Ishii et al., 1999; Paz et al., 1999; Lammert et al., 1997a; Lammert et al., 1997b; Basu and Srivastava, 1999; Reits et al., 2003). Another possible mechanism is through a process called 'cross-presentation'(Srivastava, 2002; Singh-Jasuja et al., 2000; Tamura et al., 1997). It is proposed that APC uptake of CRT chaperoned peptides is through receptors such as CD91(Binder et al., 2000; Basu et al., 2001), LOX1(Delneste et al., 2002), and then re-presented by class I molecules representing one mechanism of cross-priming. This is a

pathway by which peptides go from the endosome to the cytosol, and then to the ER and the secretory pathway, as immunogenicity of CRT-peptide complexes has been shown to be dependent on the presence of a functional proteasome complex and a functional TAP. The importance of receptor CD91 in taking up CRT/peptide complexes was demonstrated by Basu et al who showed that both α2 macroglobulin, a known ligand for CD91, and anti-CD91 antibodies can inhibit re-presentation of peptides chaperoned by CRT(Basu et al., 2001) .

The chaperones and folding enzymes described individually above, are members of the PLC, which consists of one TAP1/TAP2 dimer, four tapasin molecules, four MHC class I molecules, four ERp57 molecules and four CRT molecules(Sadasivan et al., 1996; Ortmann et al., 1994). PLC is believed to be the result of cooperative interactions among the components of the class I assembly machinery. Tapasin is the keystone of the PLC(Momburg and Tan, 2002). It bridges class I molecules to TAP(Tan et al., 2002). Furthermore, tapasin and ERp57 form a stable disulfide-linked dimer within the PLC(Peaper et al., 2005; Dick et al., 2002; Lindquist et al., 1998), which is independent of CRT and calnexin(Peaper et al., 2005). Disruption of this disulfide bond by mutation of cysteine 95 in tapasin results in incompletely oxidised MHC class I molecules, which exit the ER in an unstable conformation(Dick et al., 2002). Additionally, ERp57 interacts with the tip of the CRT P domain(Leach et al., 2002; Ellgaard et al., 2001). Both ERp57 and CRT non-covalently associate weakly with class I(Oliver et al., 1997; van der Wal et al., 1998; Kang and Cresswell, 2002). Previously, It was suggested that ERp57 was recruited into the PLC by its association with CRT or CNX. However, Gao and colleagues studied the formation of PLC in CRT deficient cells, and found that abundant ERp57 coprecipitated with TAP, even in cases there was no detectable canexin inside the PLC. They, therefore, suggested that the incorporation of ERp57 into the loading complex is not a consequence of its association with CRT or calnexin(Gao et al., 2002). Additionally, Peaper et al have showed that all the tapasin in β2m or MHC class I deficient cells was disulfide-linked to ERp57, implying that ERp57 interaction with PLC is independent of the recruitment of class I molecules(Peaper et al., 2005). Now it is

believed that ERp57, together with TAP and tapasin, forms the core, unchanging component of PLC, and that CRT as well as the MHC class I-β2m dimers cycle through the loading complex during peptide loading(Cresswell et al., 2005).

The exact functional role of each component of PLC is difficult to identify given that the interactions between the molecules are highly co-operative. In addition, ER has a powerful and redundant system to assist protein folding. If access to one chaperone is prevented, a different one can take over. Nonetheless, one approach towards understanding the nature of interactions among components of PLC and their functions involves studying the phenotype of class I complexes assembled from mutant class I molecules with altered binding to other components of PLC. For example, a non-conservative point mutation at position 134 of the HLA-A2 HC (A2T134K) renders the A2 molecule unable to bind tapasin, TAP and CRT. As a result, A2T134K/β2m complexes are rapidly exported to the cell surface in an unstable, peptide-receptive form(Peace-Brewer et al., 1996; Lewis et al., 1996; Lewis and Elliott, 1998). Another example is the mutation of asparagine at position 86 (N86). Mutagensis of N86 of HLA-B27 prevented incorporation of the HC/β2m dimer into the PLC, so there is no association of HC with tapasin, TAP or CRT. Although class I surface expression was comparable to the wild type B27, a great proportion of class I complexes were thought to be empty. Presumably the mutation of N86 prevented class I HC from glycosylation, which in turn disrupted the interaction of HC with CRT as studies have shown that the N86 is critical for the interaction of HC with CRT(Harris et al., 2001). Taken together, these data have shown that there is a correlation between the ability of class I molecules to interact with PLC and the efficiency of optimal peptide loading(Hill et al., 1993).

The relative physiological importance of the different components of PLC for antigen presentation in vivo can be assessed by using knock-out mice for each component. All components of PLC have been deleted in mice, and the strongest effect on the expression of class I/peptide molecules is obtained from mice deficient in β2m. Cell surface HC expression is below the detection limit by fluorescent-assisted sorting(Williams et al.,

1989; Zijlstra et al., 1990). The next component in the order of importance for class I expression is TAP1/TAP2 complex. Cells from TAP1 deficient mice show a 90-fold reduction in surface class I expression(Van Kaer et al., 1992). Tapasin is also important for the surface class I expression, about 10-fold reduction in surface class I expression was observed in lymphocytes lacking tapasin expression(Garbi et al., 2000; Grandea, III et al., 2000). Lastly, MHC class I expression is less critically dependent on CRT and ERp57, with threefold to fourfold reduction of surface class I for CRT deficient fibroblasts(Gao et al., 2002) and twofold decrease in class I expression in lymphocytes from ERp57$^{-/-}$ mice(Garbi et al., 2006). Until now, although the function of TAP in transporting peptides across the ER membrane has been established, the contribution of other components in the PLC to peptide loading is less well understood and still remains a topic of considerable interest.

Finally, it needs to be pointed out that, although peptide-binding onto class I molecules within the PLC in the ER contributes the large majority of class I/peptide complexes at the cell surface, many human leukocyte antigen-B (HLA-B) alleles, such as HLA-B13, B44 and B60, do not use the PLC for peptide acquaintance(Lewis et al., 1996), implying that peptides find their own MHC class I molecules outside the PLC. This has been suggested to have important evolutionary significance as many viruses, such as herpes simplex virus and adenovirus, have evolved remarkable mechanisms to inhibit MHC class I expression by interfering with the function of PLC(Lybarger et al., 2005). For example, The E3/19K protein of adenovirus was shown to impair the association of class I molecules to the PLC(Bennett et al., 1999). Thus, it has been proposed that class I molecules and alleles that are less dependent on the PLC for expression arose in response to the selective pressure exerted by microbial pathogens that subvert class I antigen presentation(Peh et al., 1998).

1.2.1.6 ER aminopeptidases associated with antigen processing

Due to the structure of the class I peptide binding groove, peptides suitable for presentation generally must have a length of 8-11 amino acid residues in

length(Rammensee et al., 1995). But evidence suggests that cytoplasmic proteolysis by proteasomes generates only the correct carboxy terminus of antigenic peptides suitable for presentation by class I molecules, and most of these peptide precursors have an extended N-terminus(Kloetzel, 2001; Cascio et al., 2001). Moreover, TAP has been shown unable to transport peptides containing a proline residue at position 2 (X-P-X_n)(van Endert et al., 1995), but up to 20% of MHC class I molecules actually present X-P-X_n peptides (Rammensee, 1997. MHC Ligands and peptide Motifs 1-457 (Landes Bioscience, Austin, Texas)). To resolve this paradox, it was proposed that X-P-X_n peptides could be transported as N-terminally extended intermediates, which can be trimmed further in the ER to yield the final peptides presented by class I molecules.

ER aminopeptidase associated with antigen processing (ERAAP in mice; human ERAAP ortholog was named ERAP-1 to designate it as the first member of an ER aminopeptidases family)(Serwold et al., 2001; Serwold et al., 2002; York et al., 2002; Saric et al., 2002; Tanioka et al., 2003) and ERAP-2 (the second ER aminopeptidase discovered in human)(Tanioka et al., 2003; Saveanu et al., 2005; Fruci et al., 2001) were identified as aminopeptidases in the ER to customize peptides for MHC class I molecules. ERAP-2 is approximately 50% identical to the amino acid sequence of ERAAP/ERAP-1 and exists in human. Mouse ortholog of ERAP-2 has not been found in the sequence database. Both ERAP-1 and ERAP-2 are up-regulated by interferon-γ (IFN-γ), which also stimulates the induction of other components of the antigen-processing pathway, such as class I molecules, TAP, and three proteasome β-subunits(Gaczynska et al., 1993; Gaczynska et al., 1994; Gaczynska et al., 1996). Furthermore, ERAP-1 and ERAP-2 colocolize with several ER markers such as calnexin and Bip(Serwold et al., 2002; Saric et al., 2002; Saveanu et al., 2005). Consistent with their localization in the ER, these enzymes are EndoH sensitive. Functionally, both ERAP-1 and ERAP-2 could remove N-terminal residues from the peptide precursors(Tanioka et al., 2003).

Human ER is equipped with both ERAP-1 and ERAP-2, which might act in a concerted fashion to provide a complete array of trimming activity in the human ER to make sure that N-extended versions of antigenic peptides are trimmed until they acquire the optimal

length to be loaded onto class I molecules(Saveanu et al., 2005). ERAP-1 prefers longer peptide substrates than 8 or 9 amino acids, and trims longer substrates to 8 or 9 residue products, which suggests that ERAP-1 trims peptides to ideal substrates for MHC class I molecules(Saric et al., 2002; York et al., 2002). Moreover, ERAP-1 preferentially removes large hydrophobic residues such as Leu and is unable to trim peptides with a proline residue at position 2(Serwold et al., 2001), which, as mentioned before, is used as a preferred anchor residue for various MHC class I molecules. In contrast, ERAP-2 has a strong preference for the basic residues Arg and Leu(Saveanu et al., 2005; Tanioka et al., 2003; Hattori et al., 2000). In an elegant study, Saveanu and colleagues have showed that several N-terminal amino acids, which cannot be removed by ERAP-1, were trimmed efficiently by ERAP2. Additionally, they found that ERAP-1 and ERAP-2 can form homodimers and heterodimers(Saveanu et al., 2005).

Although there is no evidence that ERAP-1 and ERAP-2 form part of the PLC, there is plenty of evidence that they contribute to the generation of suitable class I ligands and seem to be an integral part of the antigen-processing pathway. For instance, reducing ERAAP/ERAP-1 expression through RNA interference or gene knockout prevents the trimming of peptides for MHC class I molecules and greatly reduces the expression of MHC class I on the cell surface. In addition, a greater proportion of MHC class I molecules in ERAAP deficient cells are loaded with suboptimal paptide, which suggests that ERAAP is essential for the generation of the normal repertoire of processed peptides(Serwold et al., 2002; Hammer et al., 2006). Conversely, over-expression of ERAP1 can enhance processing in the ER of an N-terminal extended ovalbumin-derived precussor and presentation of mature epitopes(Saric et al., 2002). In addition to generating optimal peptides for class I molecules, ERAAP can also over-process peptides. Consequently, wheareas some epitopes are poorly presented in the absence of ERAAP, some are presented better(York et al., 2002; Hammer et al., 2006).

1.2.2 Production and provision of peptides for MHC class I

The origin of many peptides for display by MHC class I molecules is from defective ribosome products (DRiPs), which are newly synthesized polypeptides degraded cotranslationally or very swiftly after translation, possibly as the result of protein misfolding, misassembly, or mistranslation/transcription such as cryptic translation products(Reits et al., 2000; Schubert et al., 2000; Yewdell et al., 1996). Cryptic translation refers to polypeptides encoded by introns, intron/exon junctions, 5' and 3' untranslated regions, and alternative translational reading frames(Shastri et al., 2002). This pool of DRiPs is considerable, ranging from 20 to more than 70% of cellular translation products, depending on the cell type(Princiotta et al., 2003). Support for the theory that antigenic peptides are derived from the newly synthesized proteins came from studies using a tetracycline-regulated LCMV nucleoprotein (NP) that allows rapid cessation of protein synthesis(Khan et al., 2001). Antigen presentation stopped 48 hours after terminating NP synthesis, suggesting the antigenic epitopes were derived from newly synthesized NP. This ongoing degradation of newly synthesized proteins has the important function providing a continuous supply of peptides that are loaded onto MHC class I molecules to be presented on the cell surface(Yewdell and Bennink, 2001; Turner and Varshavsky, 2000). In cells infected by viruses, the detection of DRiPs-derived peptides may allow T cells to deal with them rapidly, without having to wait for degradation of mature protein. In healthy cells, the peptides are derived from normal cellular proteins, and the immune system is rendered tolerant to these peptides during development except in autoimmune diseases. Furthermore, complexes of self-peptide and MHC class I molecule are necessary to establish the repertoire of TCR. Another source of peptides for display by MHC class I molecules is from proteins at the end of their useful life, which are also processed to produce peptides for MHC class I, but how this is monitored is unclear, possibly some form of unfolding acts as an initiator.

The final peptides required for loading onto class I molecules are produced in several steps that occur both in the cytoplasm and in the ER. The first step involves the recognition of a protein (self or foreign) by intracellular degradation machinery and the

transfer of multiple ubiquitin moieties to the substrate protein(Hershko and Ciechanover, 1998). Complete polyubiquitination of a substrate protein is necessary for recognition by proteasome, which is a dominant protease complex and plays the major role in normal turnover of proteins in the cytosol(Kloetzel and Ossendorp, 2004; Forster and Hill, 2003). In the absence of proteasome, MHC class I molecules remain in the ER, starved for peptides due to the reduced protein degradation(Rock et al., 1994; Hughes et al., 1996). However, in vivo experiments suggest that the proteasome is the only candidate to generate the correct C-terminus of MHC class I ligands(Craiu et al., 1997; Mo et al., 1999; Yewdell et al., 2003), and the majority of the peptides generated by proteasomes are N terminal extended peptide precursors longer than 15 amino acids(Cascio et al., 2001; Paz et al., 1999; Serwold et al., 2001; Goldberg et al., 2002; Reits et al., 2004). Such NH_2-extended peptides appear to be more resistant to degradation of cytosolic peptidases than mature epitopes(Goldberg et al., 2002; Mo et al., 1999) and they may also be preferentially transported by TAP(Lauvau et al., 1999; Serwold et al., 2001).

NH_2-extended peptide precursors have to be further trimmed to mature epitopes for the loading of class I molecules, which can occur in the cytosol and/or in the ER. In the cytosol, a few cytosolic aminopeptidases have been shown to be implicated in the generation of peptides for MHC class I by trimming the extended N-terminus. Tripeptidyl peptidase II (TPP II) has been demonstrated to play a role in antigen processing by removing 2-3 N-terminal residues at a time from long peptides, unless it finds a N-terminal proline(Geier et al., 1999; Kloetzel and Ossendorp, 2004; Rock et al., 2004). It prefers substrates longer than 15 amino acids in length(Reits et al., 2004), which represent most of the proteasomal products in vivo. The importance of TPP II in the class I pathway was demonstrated by Reits et al who showed that by short interfering RNA of TPP II resulted in a marked downregulation of class I expression at the cell surface(Reits et al., 2004). Most short proteasome and TPPII products (8-16 amino acids) are degraded by thimet oligopeptidase (TOP)(Ray et al., 2004) or by aminopeptidases such as leucine aminopeptidase (LAP)(Saric et al., 2004; Beninga et al., 1998) and puromycin-sensitive aminopeptidase (PSA)(Stoltze et al., 2000). However, Levy and colleagues have demonstrated that not all class I ligands have to undergo trimming in the cytosol(Chapatte

et al., 2004). Studies have also indicated that proteasomes and the peptidases can also destroy some potential MHC class I-binding peptides by cleavage(Oliveira et al., 2001; Saric et al., 2004). In fact, more than 99% of the cytosolic peptides are destroyed within 1 min after their generation by a large number of cytosolic peptidases before they encounter TAP(Reits et al., 2003). Interestingly, some studies have suggested that the peptide precursors are bound to and protected by chaperones such as tailless complex polypeptide-1 (TCP-1) ring complex (TRiC) or chaperonin-containing TCP-1 (CCT) prior to TAP transport(Kunisawa and Shastri, 2003). Shastri et al examined the TRiC immunoprecipitates and found the presence of several distinct N-terminally extended peptides. Furthermore, when the expression of TRiC was knocked down by RNA interference, the same set of peptides were lost in the cell extracts. Additionally, cells in which TRiC was knocked down expressed a lower level of MHC class I molecules on the surface(Kunisawa and Shastri, 2003; Dunn et al., 2001). However, Reits and colleagues have studied the behavior of peptides in living cells and concluded that the majority of peptides are free rather than associated with larger protein chaperones, or if they do associate with chaperones, they probably do so in a highly transient manner(Reits et al., 2003).

1.2.3 Peptide loading and optimization

Peptide loading onto MHC class I molecules is a prerequisite for both the stability and the function of class I in presenting intracellular antigens to CTLs(Neefjes et al., 1993; Townsend et al., 1989). Optimal peptides for MHC class I heterodimers are normally 8-9 amino acids in length with specific anchor residues(Falk et al., 1991; Falk et al., 1990; Matsumura et al., 1992; Bouvier and Wiley, 1994). Optimization of the peptide repertoire presented by MHC class I molecules relates to the preferential selection of peptides with slow off-rate and undergoes time-dependent. Loading of MHC class I with highly stable peptides requires a fully competent loading complex where TAP, tapasin, CRT, ERp57 and class I molecules all interact with one another(Tan et al., 2002). Accordingly, disruption of any interactions between different the component results in compromised peptide loading. For example, Dick et al showed that peptide loading of HLA-B44 was

less optimal when the formation of the tapasin-ERp57 disulfide bond was inhibited(Dick et al., 2002). Moreover, Lewis and colleagues found that class I-TAP interaction is obligatory for the presentation of optimal peptides since a mutant HLA-A2 molecule where threonine at position 134 in the α2 domain is changed to lysine (T134K) is rapidly released to the cell surface, but is unstable due to its inability to interact with TAP(Lewis et al., 1996).

It has been proposed that peptide loading and optimization occur in two steps, the first being the loading of low-affinity peptides for stabilization of the MHC class I heterodimer, followed by replacement of low-affinity by high-affinity peptides, which induces conformation change of MHC class I molecules and dissociation of class I from loading complex(Lehner and Cresswell, 2004; Paulsson et al., 2001). Support for such two-step peptide optimization view comes from a study by Marguet et al who used GFP-tagged MHC class I to show that peptide-loaded class I can also be retained in the ER for optimizing the peptide cargo(Marguet et al., 1999). Lewis and colleagues also provided evidence that MHC class I assembly is a two-stage process. They demonstrated that in TAP deficient cells, the majority of T134K molecules do not exit the ER in comparison to the majority of T134K molecules exit in a peptide-receptive state in TAP competent cells. They suggested that supply of TAP-derived peptide is essential to allow T134K molecules to escape degradation and to exit the ER, which suggests that the mutation in T134K acts to prevent class I molecules from optimizing the peptide ligand, rather than preventing peptide binding per se(Lewis et al., 1996; Lewis and Elliott, 1998). Finally, it was also suggested that there might be an important quality control check-point between these two steps, which ensures both the retention of suboptimally loaded class I molecules and the release of those with longer half-life to the cell surface(Elliott and Williams, 2005).

Peptide optimization is tapasin-dependent. Tapasin, acting in co-ordination with other members of the PLC, has been shown to retain empty class I molecules in the ER(Grandea, III et al., 2000), mediate preferential selection of peptides with slow off-

rate, and promote the optimal peptide loading of MHC class I molecules(Grandea, III and Van Kaer, 2001; Garbi et al., 2000). In the presence of tapasin, there is a kinetic, quantitative, and qualitative improvement in the peptide repertoire of MHC class I molecules(Williams et al., 2002a; Howarth et al., 2004). Studies using peptide-sensitive antibodies have demonstrated different peptide repertoire profiles across tapasin-positive and tapasin-negative cell lines(Tan et al., 2002; Purcell et al., 2000). Additionally, peptide-elution studies of MHC class I molecules in the presence or absence of tapasin have found a reduction in the recovery of peptides from tapasin negative cell lines(Garbi et al., 2000; Zarling et al., 2003). Furthermore, peptide optimization occurs preferentially at the TAP interface. It was reported that in the presence of soluble tapasin (C-terminal deletion), which can bind to class I but cannot bind TAP, surface expression of HLA-B44 and HLA-B27 were restored but with optimization peptide profiles that were intermediate between no tapasin and full length tapasin(Williams et al., 2002b). Finally, it has been suggested that the dependency of peptide optimization on tapasin is allele specific. For example, Williams et al have shown that in the absence of tapasin, HLA-B2705 could load peptides and also improve peptide cargo over time, but only a very small number of HLA-B4402 class I complexes assembled with most retained in the ER and subsequently degraded due to the inability to optimize the peptide cargo(Williams et al., 2002b).

Tapasin has also been suggested to contribute to peptide optimization by retrieving of suboptimally loaded MHC class I molecules from the Golgi network to the ER by its association with COPI(Paulsson et al., 2002). Previously, it has been generally assumed that only MHC class I molecules loaded with peptides exit the ER. However, studies by Baas et al found that some peptide-receptive class I molecules or perhaps more correctly class I complexes bound to suboptimal peptides may exit the ER and travel along the secretory pathway. These suboptimally loaded class I molecules could potentially recycle between the Golgi and the ER(Hsu et al., 1991). COPI-coated vesicles ferry cargo molecules from the Golgi to the ER and are recruited by carboxy-terminal double lysine motif containing proteins(Letourneur et al., 1994; Schekman and Orci, 1996; Rothman and Wieland, 1996). Tapasin is a transmembranne protein with a double lysine motif in its carboxyl-terminal cytoplasmic tail(Li et al., 1997; Ortmann et al., 1997). Wang et al

have demonstrated that tapasin-associated, peptide-receptive class I molecules were found throughout the early secretory pathway and proposed that tapasin-associated unstable MHC class I molecules that fail to load optimal peptide and escape the ER are allowed a second chance by recycling between the ER and the Golgi complex until optimal peptide is loaded(Paulsson et al., 2002).

In addition to tapasin, a second component that complements the function of tapasin for MHC class I peptide optimization within the PLC has been proposed. This second component would relate to the recycling of MHC class I molecules loaded with suboptimal peptides, and may involve the CRT/UDP glucosyl glycoprotein transferase (UGGT) binding cycle that may lead to an iterative process of peptide optimization(Wright et al., 2004). Studies have found that while some MHC class I complexes loaded with optimal peptides traffic through the secretory pathway to the cell surface, it is possible the suboptimal peptides will dissociate from class I soon after leaving the PLC, thereby regenerating the flexible HC conformation. Furthermore, as described above, tapasin-associated unstable MHC class I molecules that fail to load optimal peptide and escape the ER are allowed a second chance to recycle between the ER and the Golgi(Paulsson et al., 2002). These unfolded MHC class I molecules might confer recognition by UGGT, which has been shown to recognize a localized disruption in conformation(Caramelo et al., 2003). One region predicted to be mobile in the absence of peptides resides at one end of the α1 helix around the glycosylation site at Asn86(Zacharias and Springer, 2004). Recognition by UGGT would lead to glucosylation of the core glycan and restoration of the CRT-binding site. Relevant to this idea are the observations that MHC class I molecules in the CRT deficient cells have a faster trafficking rate and a reduced steady-state surface expression that could be rescued with exogenous peptide(Gao et al., 2002). Additionally, Kearse et al have demonstrated that murine class I complexes released from PLC can be blocked by using inhibitors of deglucosylation(van Leeuwen and Kearse, 1996a). Taken together, CRT may aid the retention of the suboptimally loaded class I complexes in the ER for peptide optimization(Elliott and Williams, 2005). Therefore, it has been suggested that tapasin is

the key chaperone that selecting the peptides with a long off-rate for binding of class I molecules with contribution from CRT.

Optimization of peptide loading onto class I complexes may be linked to their retention time in the ER. Lewis et al have tested this idea by incubating cells expressing T134K molecules in the presence of the drug brefeldin A (BFA), which promotes the retention of newly synthesized class I in the ER. They found that the fraction of stable T134K molecules increased dramatically(Lewis and Elliott, 1998), and they proposed that ER retention alone may facilitate the assembly of class I molecules with optimal peptides. Consistent with this model, the importance of tapasin for peptide optimization as described above may be due to its ability in retaining the empty class I molecules in the ER until they acquire the optimal peptide(Grandea, III et al., 2000).

The exact molecular mechanism for optimizing the peptide cargo displayed on class I molecules is not yet fully understood. It could be achieved by peptide exchange, as described above(Sijts and Pamer, 1997). This process may require the assistance of a cofactor similar to the role of HLA-DM, which edits the peptides bound to MHC class II according to their binding stability(Karlsson, 2005). Tapasin, like DM, appears to retain and stabilize class I molecules in a peptide-receptive stage and has been suggested to have the model of action similar to the quality control model proposed for DM(Brocke et al., 2002). Further studies are needed to ascertain the role of tapasin or other cofactors in facilitating peptide loading and selecting optimally binding peptides.

1.2.4 Export of MHC class I to the cell surface

Upon peptide loading and optimization, peptide/MHC complexes dissociate from the TAP complex, exit the ER, and then transport to the plasma membrane via Golgi apparatus(Ortmann et al., 1994; Suh et al., 1994). However, the mechanism for the export of class I molecules from ER is not very clear. It has been assumed that class I molecules exit ER in vesicles in bulk flow, which proposes that retention and retrieval signals keep

ER resident proteins in the ER while cargo proteins (membrane and secretory proteins) progress by default to the Golgi system(Wieland et al., 1987). However, the transport rate of peptide loaded MHC class I complex from ER to Golgi remains unchanged after dissociation from TAP. Furthermore, peptide loaded MHC class I molecules accumulate at ER exit sites from which TAP molecules are topologically excluded. On the basis of these observations, a second model, in contrast to the bulk flow model, has been proposed that cargo proteins contain signal sequences that mediate selective incorporation into COPII-coated vesicles(Kuehn and Schekman, 1997; Spiliotis et al., 2000). MHC class I molecules, however, do not possess any of the known ER export sequences. Thus, recent models favor a cargo receptor-mediated transport of class I molecules out of the ER. This model suggests that B cell-associated protein (Bap) 31, and perhaps Bap29, ER molecules identified as putative cargo receptors, are recruited to the peptide-loading complex in which they interact directly with MHC class I and possibly with tapasin. This association promotes the targeting and possibly the clustering of class I at ER exit sites. At the time of packaging into coat protein complex II (COPII)-coated vesicles in the ER(Rothman and Wieland, 1996; Schekman and Orci, 1996) or upon arrival at the Golgi, stably assembled class I molecules are released from Bap31 and transport to the cell surface(Paquet et al., 2004). In this way, MHC class I molecules with high affinity for transport receptors are positively selected for efficient export from ER(Spiliotis et al., 2000). Support for this model also comes from the study showing that, in the absence of Bap29/31, there is a loss co-localization of MHC class I with mSec31 (p137), a component of mammalian COPII coats, which associates with a delay in class I traffic from ER to Golgi(Paquet et al., 2004).

Upon reaching the cell surface, MHC class I molecules are susceptible to internalization. By tagging the HLA-A2 HC with GFP, Gromme and colleagues have demonstrated that a small fraction of these class I molecules were found in endo/lysosomal compartments, and these HLA-A2-GFP molecules were not directly derived from the biosynthetic pathway because they were resistant to BFA treatment. They suggested that these class I molecules were routed from the cell surface into the endocytic pathway where they can be reloaded with peptides generated in the endocytic compartments from exogenous

antigens(Gromme et al., 1999). This has been regarded as one of the mechanisms for cross-presentation(Rock and Shen, 2005). Evidence for recycling MHC class I molecules also comes from other studies, which showed that surface MHC class I molecules can be internalized into endosomal vesicles where peptide exchange occurred(Dasgupta et al., 1988; Chiu et al., 1999).

In the above three sections, I have discussed the production and provision of class I peptides, peptides loading onto class I molecules and their optimization and export of peptide-loaded MHC class I molecules to the cell surface. Despite the highly efficient generation of peptides by proteasomes, only a small fraction of these peptides presented by class I molecules are displayed on the cell surface for recognition by CD8 T cells. The efficiency of the MHC class I antigen presentation pathway is low, but exactly how many protein molecules have to be degraded to produce a single MHC/peptide complex on the cell surface? By using a monoclonal TCR-like antibody, 25-D1.16, which is specific for ovalbumin-derived epitope SIINFEKL/H-2K^b complex, Princiotta and colleagues have calculated that between 440 and 3000 protein molecules are degraded to generate each surface K^b/SIINFEKL complex, an average efficiency of 0.05%(Princiotta et al., 2003).

1.2.5 CD8$^+$ T cell activation and effector function

Following the export from the ER, peptide-loaded class I molecules traffic to the cell surface for inspection by CD8$^+$ T cells. CD8$^+$ T cells use T cell receptors (TCRs) to recognize antigen/MHC complexes. TCRs are cell surface heterodimers consisting of either disulfide-linked α- and β- or γ- and δ-chains. Most T cells express $\alpha\beta$ TCRs. Compared with $\alpha\beta$ TCRs, much less is known about $\gamma\delta$ TCRs, which will not be discussed here. The $\alpha\beta$ TCR is associated physically with a series of polypeptides, collectively called CD3, which comprises 4 invariant polypeptides, called γ, δ, ε and ζ. An alternatively spliced form of ζ, called η, has also been identified. The CD3 association is required for the expression of the TCR complex at the cell surface and signal transduction following antigen recognition by the TCR heterodimer. $\alpha\beta$ TCRs bind

generally with very low affinity to peptide-MHC (pMHC) complexes, and the half –life of the binding of the TCR to pMHC is seconds at 37°C(Davis et al., 1998; van der Merwe and Davis, 2003). Activation of CD8 T cells can be induced by recognition of as few as 10-100 MHC/peptide complexes displayed at the cell surface(Yewdell et al., 2003; Sykulev et al., 1996; Christinck et al., 1991; Brower et al., 1994). But exactly how many interactions between TCRs and pMHCs are necessary for activation of CD8 T cells may depend on the type of the T cell and its activation state. For instance, naïve T cells are less sensitive to antigen stimulation than CTLs; murine hybrids (derived from the fusion of a normal T cell with a T-cell tumour cell) are known to be easily triggered(Stoll et al., 2002; Hugues et al., 2004).

Recognition of ligands (pMHCs) by $CD8^+$ T cells induces a conformational change in TCR-CD3ε complex, which exposes a C-terminal proline-rich sequence in its cytoplasmic tail that requires the adapter molecule for down-stream signalling to initiate early T cell activation(Krogsgaard et al., 2003). These signals activate specific transcription factors, which then translocate to the nucleus to activate genes including 'immediate early genes' for cell division and cytokine secretion, such as IL-2 and IFN-γ(Huppa et al., 2003).

Activated CTLs can kill target cells by two different mechanisms. One mechanism is via granule exocytosis. CTLs contain granules within their cytoplasm. These granules contain a number of proteins. Two important proteins are perforin and granzymes, which are a collection of serine proteases. After binding to its target cell, CTL direct its granules towards the membrane adjoining the target cell. Then, the granule contents are discharged into a cleft between CTL and target cell. Perforin is able to form pores in the target cell membrane, through which Granzymes are thought to enter the target cell to cleave proteins. In this way the target cells is lysed. Another mechanism that activated CTL uses to kill target cell involves the Fas pathway. Fas is a death molecule which can directly signal apoptosis. Fas can be expressed on many cell types. The ligand for Fas (Fas-L) is expressed on mature $CD4^+$ and $CD8^+$ T cells after activation. Binding of TCR to its specific pMHC causes expression of Fas ligand (Fas-L) on CTL. Fas on the target cell

will be cross-linked by the Fas-L on the CTL. Then, apoptosis and death of the target cell will be triggered. Therefore, by displaying the short peptides on the cell surface for recognition by CTLs, cells carrying foreign or mutated proteins can be eradicated(Shastri et al., 2002).

1.2.6 Objective of the project

CRT has been regarded to be a multi-compartmental and multifunctional protein. That one protein can perform so many functions is intriguing and further investigation is required in order to fully understand the CRT functions. In particular, we still do not understand what exact functional role CRT plays in MHC class I assembly and antigen presentation, although there is definitely impaired surface class I expression in a calreticulin deficient cell line. The aim of this project is to try to investigate the following outstanding issues:

(1) What's the functional role of CRT in MHC class I antigen presentation?

(2) As a general chaperone for the folding and assembly of newly synthesized glycoproteins, does the absence of CRT affect the expression and/or function of the other components of PLC?

(3) How does CRT contribute to MHC class I antigen presentation?

(4) How does the function of CRT relate to its structure?

To study the conventional MHC class I pathway, the protein antigen of interest has to be expressed inside cells. In chapter 3 a novel method was described to deliver protein antigen directly into the cell cytosol. Mouse fibroblasts, RMA and its TAP-2 deficient counterpart cell line-RMA-S were used to demonstrate that both adherent and suspension cells were permeabilized with streptolysin O (SLO) and chicken ovalbumin (OVA) was successfully introduced into the cytoplasm. Evidence was presented that in TAP competent RMA and mouse fibroblast cells OVA was properly processed and OVA-derived peptide $OVA_{258\text{-}265}$ (or SIINFEKL) was successfully presented via MHC class I molecule (K^b) to SIINFEKL- K^b specific T cell hybridoma-B3Z, while TAP-2 deficient RMA-S cells failed to stimulate B3Z.

To investigate the functional role CRT plays in MHC class I antigen presentation, in chapter 4 the SLO antigen delivery system was used to introduce OVA into the cytoplasm of both CRT deficient mouse fibroblasts (K42) and CRT competent cells (K41 (CRT+/+), and K42 cells transfected with full length human CRT. It was showed that CRT deficient cells exhibited a TAP deficient phenotype in terms of class I assembly, without loss of TAP expression or functionality; expression of calnexin, tapasin was unchanged while up-regulation of ERp57 was observed in CRT deficient cells; a higher concentration of antigen in the cytosol was required for specific T cell stimulation in CRT deficient cells than in CRT competent cells. The results indicated that CRT has a functional role in the maintenance of the low peptide concentration threshold required in the ER for efficient antigen presentation to T cells.

To learn more about how CRT contributes to MHC class I antigen presentation and how the function of CRT relates to its structure, in chapter 5 full length CRT or one of three structurally and functionally distinct domains, N-domain, the central P-domain, and the C-domain, were introduced into both CRT deficient cells (K42) and CRT competent cells (K41) to examine the role of CRT and its truncated forms in the formation of the PLC and MHC class I antigen presentation. It was found that while full length CRT restored MHC class I surface expression on K42 (CRT-/-) cells to the levels seen in the wild-type K41 (CRT+/+) cells, the expression of P-, NP-, PC-domains of CRT down-regulated MHC class I expression in K42 (CRT-/-) cells. In contrast, K41 (CRT+/+) cells transfected with truncated CRT exhibited a normal class I surface expression, indicating that disruption to MHC class I assembly only happened in the absence of CRT. Assessment was also made on the effect of truncated CRT expression on cellular functions, such as peptide loading, TAP function, and expression of other members of PLC. The data indicated that in cells without CRT calnexin may compensate for the loss of CRT function. In CRT deficient cells, expression of truncated forms of CRT might block the redundant function of calnexin, leading to further reduced cell surface MHC class I expression.

Chapter 2 Materials and Methods

2.1 Cells and antibodies

Table 2.1: Cells used in this study

Cell line	Description	Defect	Source
K41	Mouse embryo fibroblasts(Nakamura et al., 2001b), expressing H-2K^b and H-2D^b		Dr M Michalak
K42	Mouse embryo fibroblasts (Nakamura et al., 2001b), expressing H-2K^b and H-2D^b	CRT deficient	Dr M Michalak
EG7-OVA	A transfected clone of T lymphocyte, producing chicken ovalbumin(Moore et al., 1988), expressing H-2K^b		Alain Townsend
RMA	Derived from a Rauscher leukaemia virus-induced cell lymphoma(Karre et al., 1986), expressing H-2K^b and H-2D^b		Alain Townsend
RMA-S	Derived from a Rauscher leukaemia virus-induced cell lymphoma(Karre et al., 1986), expressing H-2K^b and H-2D^b	TAP-2 mutant cell that express low levels of class I molecules H-2K^b and H-2D^b (Ljunggren and Karre, 1985; Attaya et al., 1992)	Alain Townsend
R1E/TL8x	Mouse B cell line, expressing H-2K^b	β2m deficient	The American Type Culture Collection (ATCC, USA)

B3Z	T cell hybridoma specific for H-2K^b-SIINFEKL complex. This hybridoma has a copy of β-galactosidase gene under the Il-2 promotor, which is activated following T cell receptor engagement(Karttunen et al., 1992)		Dr N. Shastri
Mycl-9E10	A hybridoma cell line producing anti human c-myc antibody		ATCC
25-D1.16	A hybridoma cell line producing monoclonal antibody specifically against H-2K^b/SIINFEKL complex(Porgador et al., 1997)		Dr Germain
Y3	A hybridoma cell line producing monoclonal antibody reactive with mouse MHC class I H-2K^b		ATCC

The above cell lines were grown in RPMI 1640 supplemented with 10% FCS and L-Glutamine (Gibco BRL). Transfected K41, k42 cells were cultured in the same medium with 6mg/ml G418 (SIGMA).

Table 2.2: Antibodies for FACS

Antibody	Target	Concentration, Dilution	Source, type	Conjugation
Y3	Mouse class I: H-2K^b	Unknown protein G purified hybridoma supernatant, 1/200	Hybridoma supernatant, of Y-3 cell line, Mouse, monoclonal	None
25-D1.16	H-2Kb/SIINFEKL complex	Unknown protein G purified hybridoma supernatant, 1/200	Hybridoma supernatant, Mouse,monoclonal	None
Anti-mouse PE (Sigma)	Mouse IgG	Unknown, 1/200	Sheep, polyclonal	PE

Table 2.3: Antibodies for Western Blot

Antibody	Target	Concentration, Dilution	Source, type	Conjugation
Anti-myc	Myc-tagged recombinant proteins	Unknown protein G purified hybridoma supernatant, 1/3,000	Hybridoma supernatant, of myc-9E10 cell line, Mouse, monoclonal (Clone: 9E10)	None
Anti-CRT serum	Human, mouse Calreticulin	Unknown, 1/3,000	Rabbit, polyclonal	None
Anti CRT (Stressgen)	Human Calreticulin	0.9 mg/ml, 1/2,000	Mouse, monoclonal	None
Anti-calnexin serum	Mouse calnexin	Unknown, 1/2,000	Rabbit, polyclonal	None
Anti-TAP2 serum	Mouse TAP2	Unknown, 1/500	Rabbit, polyclonal, provided by Professor Ping Wang	None
Anti-tapasin serum	Mouse tapasin	Unknown, 1/10,000	Rabbit, polyclonal provided by Professor Ping Wang	None
Anti-ERp57 (abcam)	Mouse ERp57	Unknown, 1/3,000	Rabbit, polyclonal	None
Anti-HC serum (T18)	Free mouse D^b and K^b HC	Unknown, 1/2,000	Rabbit, polyclonal, provided by Dr Tim Elliott	None
Anti mouse – HRP (Sigma)	Mouse IgG	Unknown, 1/10,000	Goat, polyclonal	HRP
RG96 (Sigma)	Rabbit IgG	Unknown, 1/7,000	Mouse, monoclonal (Clone: RG-96)	HRP

2.2 Protein G Purification of Antibodies

The ImmunoPure Immobilized protein G (PIERCE, Lot number: FE71154) column was washed with 5 bed volumes of 20 mM Sodium Phosphate Buffer, pH 7.0. The hybridoma supernatant (containing Y3 or anti-myc antibody) was centrifuged or filtered to remove any particulates, and then applied onto the column. The column was washed with 5 bed volumes of 20 mM Phosphate Buffer, pH 7.0. The bound antibody was eluted with 1-3 bed volumes of 0.1M Glycine into the collection tubes prepared by adding 0.1 ml of 1M Tris per ml of each fraction to be collected. Fractions were pooled and dialyzed against 3 changes of PBS, at least 100 times the sample volume. Aliquots were stored at -20°C. The column was regenerated with 2% Ethanol and stored at 2-8°C.

2.3 Peptides and proteins

SIINFEKL (ovalbumin peptide 257-284), flu matrix protein peptide GILGFVFTL, and fluorescein (FITC) tagged RRYQNSTEL as TAP transport substrate were purchased from Eurogentic, Belgium and received as lyophilised powder and re-suspended in water to a stock concentration of 5mg/ml. For routine use these were then diluted 1/10000 (final concentration 0.5μg/ml). BSA and chicken ovalbumin were purchased from Sigma.

2.4 Molecular cloning

2.4.1 DNA modifying enzymes

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

Restriction endonucleases (Promega UK): Generally 1-2μg DNA, 1-2 units of enzyme, 5μl 10x reaction buffer, 0.5μl BSA, Sterile water to a total of 50μl. Incubate at 37°C (or optimal enzyme temperature) for 1 hour.

T4 DNA Ligase (Promega UK): Generally 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 12 µl.

2.4.2 Polymerase Chain Reaction (PCR)

Reagents for PCR were mixed in a total volume of 50µl as laid out in the table below. Taq DNA Polymerase (Invitrogen) was used. PCR was carried out for 30 cycles on a GeneAmp PCR System 9700 (PE Applied Biosystems):

1) Denaturation (initial) 94°C 5 min.
2) Denaturation (cycle) 94°C 45 secs.
3) Annealment (cycle) 55°C 45 secs.
4) Polymerisation (cycle) 72°C 60 secs.
5) Polymerisation (final) 72°C 10 min.

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	5U/µl	0.2	1 unit
H_20		39.3	

The genes of P domain, PC domain, NP domain and full length human CRT were amplified using different combination of the following primers (synthesized from Sigma) with Tag DNA Polymerase (Invitrogen):

Forward-F 5'-(PstI)CTG CAG-gag cct gcc gtc tac ttc aag-3'

Reverse-F 5'- (NotI)GC GGC CGC-ggc ctg gcc ggg gac atc ttc--3'

Reverse-NP 5'-(Not I)GC GGC CGC-ata ggc ata gat act ggg atc-3'

Forward-P 5'-(PstI)CTG CAG -gat tgg gac ttc ctg cca ccc-3'

P domain: Forward-P, Reverse –NP 330 bp

PC domain: Forward-P, Reverse-F 645 bp

NP domain: Forward-F, Reverse-NP 922 bp

CRT: Forward-F, Reverse-F 1237 bp

2.4.3 TA TOPO cloning

PCR products were run on a 1% agarose gel and purified using QIAquick Gel Extraction Kit (QIAGEN) before use in the TOPO TA Cloning (Invitrogen) reaction as below:

Gel Purified PCR product 4 μl

Salt Solution 1 μl

TOPO TA vector 1 μl

These were mixed gently and incubated for 5 min at room temperature.

2.4.4 Transformation of chemically competent E. coli

Competent cells were thawed slowly on ice (~30 min) and gently resuspended. 100μl of cells was dispensed into a pre-cooled eppendorf and ~10ng supercoiled plasmid DNA or 2 μl of T4 ligation product or product from the TOPO TA cloning reaction was added. Cells were incubated with the DNA on ice for 30 min before heat shocking for 30 seconds at 42°C on a heat block (Grant QB2T). Cells were then put back on ice, topped up with 0.5 ml of prewarmed SOC (37°C), and shaken vigorously for 1 hour at 37°C to allow the bacteria to recover. 100 – 250μl of transformed bacteria were then spread on LB agar containing the appropriate antibiotic for selection. Agar plates were incubated overnight at 37°C. 2-5ml LB broth with 50μg/ml ampicilliin 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. 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.

2.4.5 Bacterial Growth

Sterilisation of Broth and agar was carried out by autoclaving at 120°C for 90 min.
Luria Broth – 16 LB capsules (Bio101) were added to 1 litre of Millipore water 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 15ml poured into 10cm Agar plates (Sterlin, UK).
SOB - 20g Tryptone, 5g Yeast Extract B, 0.5g NaCl, 5g $MgSO_4.1H_2O$ (Bio101 formulation) in 1 litre Millipore water.
SOC - filter sterilised glucose 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 Luria Broth and LB Agar as required.

2.4.6 DNA extraction and purification

DNA plasmids were extracted from E. coli strains DH5α using mini/maxiprep kits (Qiagen, UK) according to the manufacturers instructions.
DNA fragments were purified from restriction endonucleases and other molecular biology tools using a PCR purification kit (Qiagen, UK).
DNA fragments were purified from agarose gels using Qiaquick Gel Purification kit (Qiagen, UK)

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:

DNA concentration = A_{260} x 50 µg/ml x dilution factor

RNA concentration = A_{260} x 40 μg/ml x dilution factor

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.

2.4.7 Agarose Gel separation of DNA/RNA

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

Ethidium Bromide stock solution: 1g EtBr in 100ml Millipore water. (Stored in the dark at room temperature)

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 UK) was added to 10μl of DNA/RNA sample. DNA/RNA samples were then run alongside a 1Kb Ladder (New England) in TAE buffer through 1% Agarose Gels at 100V for 20-40min. Gels were stained in 200ml TAE buffer with 10μl Ethidium Bromide Stock solution and visualised using an AlphaImager system (Alpha Innotech, UK).

2.5 Establishment of stable gene expression cell lines

2.5.1 Determining G418 sensitivity

Since transfected cells are to be selected using G418 (A1720, Sigma) it is necessary to determine the drug sensitivity of the cell line to be transfected.

K42 and K41 Cells were plated at around 25% confluence on a 6-well plate. Medium at various concentrations of G418 from 2-8mg/ml was added to the cells. Fresh G418 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 G418 (6mg/ml) at which no cells survived after 10 days of culture was subsequently used to select transfected cells.

2.5.2 Cell Transfection

All plasmids were linearized at convenient restriction sites after stop codon using Sca I restriction enzyme before transfection. 2x 10^5 cells were seeded per well of a six-well or 35 mm tissue culture plate in 2 ml normal growth medium and incubated at 37°C in a CO_2 incubator until the cells were 70-80% confluent. The following solutions were prepared in 12 x 75 mm sterile tubes. Solution A: For each transfection, 2 μg plasmid DNA was diluted in 375 μl serum-free OPTI-MEM I (GiBco BRL, Lot number: 1175599). Solution B: For each transfection, 12 μl LIPOFECTAMINE Reagent (Life Technologies) was diluted in 375 μl serum-free OPTI-MEM I. The two solutions were combined, mixed gently, and incubated at room temperature for 15-45 min. Cells were washed once with 2 ml serum-free OPTI-MEM I. For each transfection, 750μl of serum-free OPTI-MEM I was added to each tube containing the lipid-DNA complexes (no antibacterial agents). The diluted complex solution was mixed gently and overlaid onto the washed cells. The cells were incubated for 5 h at 37°C in a 5% CO_2 incubator. 1.5 ml OPTI-MEM I with 20% FBS was added without removing the transfection mixture. Medium was replaced at 18-24 h following start of transfection.

2.5.3 Generating stable cell lines

16 hours post transfection, the medium was exchanged for medium supplemented with 6 mg/ml of G418 and this was used for the next 14 days. This is the time for the G418 to act on the nontransfected cells, which then detach and are washed away during the medium exchange. Once all cells have died in the well of the negative control (nontransfected), cloning was carried out.

Cells were trypsinized, spun down, resuspended in the medium containing 6 mg/ml G418, and plated onto 10cm dishes. Cells were grown for two weeks. Once the big colonies were visible by eye when viewing from the bottom of the dish, 10 colonies were picked off with an inoculation loop and transferred to separate wells of a 24 well plate containing normal medium with 6 mg/ml of G418. Cells were transferred and grown in T25 flasks. Expression of recombinant CRT was determined by western blotting using

the antibody pair: first antibody. anti-myc; second antibody. anti-mouse HRP, or anti-CRT (against human CRT, Stressgen) was used to double check the expression of recombinant CRT.

2.6 Western Blotting

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

Lysis Buffer: 1% Triton X-100 in PBS

Run Buffer: 0.025M Tris, 0.192M Glycine, 0.1% SDS (Flowgen)

Transfer Buffer: 0.02M Tris, 0.15M Glycine, 20% Methanol (Flowgen)

PBST: PBS, 0.05% Tween (Sigma)

Enhanced Chemiluminescence fluid (Pierce): 1ml Super signal Dura West luminal enhancer solution, 1ml stable peroxide buffer.

10^6 cells were centrifuged at 300g for 5 minutes, the supernatant discarded and the pellets frozen at -20°C. After thawing, cells were resuspended in lysis buffer to a final concentration of 10^7 - 10^8 cells ml^{-1} and incubated on ice for 10 minutes. Cells were then centrifuged at 13,000 rpm for 15 minutes at 4°C in a micro centrifuge (Eppendorf centrifuge 5415R) and the supernatant 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 12-14% SDS protein separating gels made using the Flowgen Protogel system (Flowgen) and run in an Xcell SureLock mini Cell and Blot module (Invitrogen) according to the manufacturer's instructions for 1 hour at 200V or until samples had travelled sufficiently. A High Molecular Weight Rainbow Marker was run alongside the samples to allow analysis of the size of the detected protein. The gel was then blotted on to Hybond C Extra membrane (Amersham) in the same module at 20V for 2 hours. The membrane was carefully removed and blocked in 10 ml of PBS 2% Marvel (Premier International Foods, UK) on a plate rocker (Stuart Gyro Rocker STR-9). The membrane was washed 3 times in PBS and transferred to 10 ml of PBST 2% Marvel milk 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 2% 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 parafilm 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.

2.7 Cell staining and FACS analysis

FACS analysis was carried out on a Becton Dickinson FACScan running CellQuest^{TM} software. Cells were gated according to size, as measured by forward scatter and side scatter. Typically 20,000 – 50,000 events were collected for each condition.

For cell staining: cells were re-suspended to a concentration of 10^6 cells ml^{-1} in cold FACS buffer and 100μl aliquots were added to wells in a round bottomed 96-well plate (Merck, Germany). The plate was centrifuged at 300g for 5 minutes, the supernatant flicked off gently and the plate vortexed to loosen the cells. The cells were then resuspended in 50μl of FACS buffer containing the appropriate antibodies at their optimal dilution (determined by antibody titration). Cells were incubated with antibody in the dark at 4°C for 30-60 min. The cells were then washed 2 times in FACS buffer by vortex, resuspension and centrifugation. Where necessary the cells were then resuspended in 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, UK) for FACS analysis.

2.8 Empty MHC class I molecules come out in the cold

Equal numbers of K41, K42, K42-P, K42-PC, K42-NP, K42-CRT, RMA and RMA-S were cultured at 26°C with 5% CO_2 for 24 h. As a control, the same number of these cells

were also cultured at 37°C with 5% CO_2 for 24h. Cells were washed, and surface MHC class I was stained with mAb Y3 (recognizes H-2K^b). Anti-mouse IgG-PE was used as secondary antibody. Cells were then analysed by flow cytometry.

2.9 Titration of vaccinia virus

Target cells ($0.5X10^6$) were infected with different amounts (2µl, 5µl, 10µl, and 25 µl) of recombinant vaccinia viruses expressing either a minigene encoding SIINFEKL with leader sequence to the ER or matrix protein of Flu as a control in 200µl PBS solution supplemented with 0.1% (w/v) bovine serum albumin at 37°C for 1 hour. The infection mixture was then transferred to a T25 flask. 2.5 ml of normal growth medium was added to each flask, and the cells incubated overnight at 37°C in a 5% CO_2 incubator. Cells were then washed, stained with mAb Y3 and 25-D1.16, a mAb with TCR-like specificities that recognizes the mouse MHC class I molecule H-2K^b and SIINFEKL peptide complex. Anti-mouse IgG-PE was used as secondary antibody. Cells were then analyzed by flow cytometry.

2.10 Streptolysin O activation

1ml of PBS supplemented with 0.05% (w/v) BSA was added to a vial containing 25,000U of streptolysin O (SLO) (S5265-25KU, Sigma). The solution was resuspended and then transferred to a "Universal" tube. 4ml of PBS supplemented with 0.05% (w/v) BSA was added to the "Universal" and mixed. 20ml of PBS was added to the "Universal" to make the final concentration of SLO 1000U/ml. SLO was activated by addition of dithiothreitol (Invitrogen) to a final concentration of 5mM. After incubating at 37°C for 2 hours, SLO was placed on ice and tested for its activity by performing a permeabilization experiment described below. If activity was satisfactory, it was aliquoted and stored at –20°C until use.

2.11 Titration of streptolysin-O

Cells ($1x10^6$) were washed twice with warm RPMI 1640 (no serum), resuspended in 200μl of RPMI 1640, and placed in the wells of a 24-well plate. Different amount of SLO were added to each well and the plate agitated immediately after each addition. Fluorescein (FITC) tagged RRYQNSTEL peptides were added to each well at a concentration of 10μM. Plates were incubated at 37°C with 5% CO_2 for 10 min, and agitated twice during this period. 500μl of warm RPMI 1640 media with 10% FCS was added to each well, and the plate was replaced at 37°C for further 30 min. Cells were washed once with ice-cold growth media and resuspended in 700μl of ice-cold growth media. 10μl 1mg/ml propidium iodide (P4170, Sigma) in PBS was added and the cells incubated on ice for 5-10min. The cells were then centrifuged and resuspended in 700μl of ice-cold growth media and analysed by 2 colour flow cytometry: colourless cells = non permeabilised; green only cells = permeabilised and resealed; red or red and green cells = dead.

2.12 T cell (B3Z) activation assay with target cells pulsed with exogenous peptides

B3Z T cell hybrids express lacZ upon binding of the T cell receptor (TCR) to SIINFEKL/ K^b complex. The amount of lacZ enzyme was quantified by the hydrolysis of CPRG (chlorophenol red β-galactoside; Calbiochem), which produces chlorophenol red absorption at 595 nm. Target cells were pulsed with 1μg/ml SIINFEKL peptide or with flu matrix protein peptide GILGFVFTL as a control for 3 hours at 37°C, then centrifuged at 300g for five minutes and the supernatant discarded. The cells were then washed 3 times with 200μl PBS by vortex, resuspension and centrifugation. The T cell hybrids (B3Z) were added into each well (10^5 cells) and co-cultured overnight at 37°C in a CO_2 incubator. Next day, individual cultures were centrifuged at 300g for five minutes and the supernatant discarded. The cells were then washed once with 200μl of PBS by vortex, resuspension and centrifugation and then lysed by addition of 100 μl Z buffer (100 mM 2-mercaptoethanol, 9 mM $MgCl_2$, 0.125% NP-40 in PBS) containing 0.12 mM CPRG. After 4 hours incubation at 37°C, 50μl stop buffer (300 mM glycine and 15 mM EDTA

in water) was added to each well and the absorption of each well was read using a 96-well plate reader. Absorption wavelength used was 570 nm with 630 nm as the reference wavelength.

2.13 Antigen presentation assay with TCR-like antibody 25-D1.16

Target cells ($0.5X10^6$) were infected with 25μl recombinant vaccinia virus expressing either a minigene encoding SIINFEKL with leader sequence to the ER or matrix protein of flu as control in 200μl of PBS solution supplemented with 0.1% (w/v) bovine serum albumin at 37°C for 1 hour. The infected cells were transferred to a T25 flask containing 2.5 ml normal growth medium and incubated at 37°C in a 5% CO_2 incubator overnight. The cells were then washed and stained by 25-D1.16 to detect K^b/SIINFEKL complex presented on the cell surface. Anti-mouse IgG-PE was used as second antibody. Cells were then analyzed by flow cytometry.

2.14 Antigen presentation assay with B3Z.

Cells ($1x10^6$) were washed twice with warm RPMI 1640, resuspend in 200μl of RPMI 1640 (no serum), and added into the wells of a 24-well plate. The cells were permeablized by adding streptolysin-O (SLO) at an appropriate concentration (10 U/10^6 cells for K41, K42 and K42-CRT; 45 U/ 10^6 cells for RMA and RMA-S). The plate was agitated immediately after each addition. Different concentrations of ovalbumin or BSA as control were added to each well. The plate was incubated in a 37°C incubator with 5% CO_2 for 10 min and agitated twice during this period. 500μl of warm RPMI 1640 media with 10% FCS was added and cells incubated for further 30 min. The cells were then washed twice with normal growth media and resuspended in growth media at $1x10^6$ /ml. 100μl of the above cell suspensions were placed in a well of a 96-well plate. EG7 was used as the experiment control. 100μl of $1x10^6$/ml B3Z cells were added and co-cultured with target cells overnight at 37°C with 5% CO_2. Individual cultures were then centrifuged at 300g for five minutes and the supernatant was discarded. The cells were

then washed 1 time with 200 μl of PBS, lysed by addition of 100 μl Z buffer (100 mM 2-mercaptoethanol, 9 mM $MgCl_2$, 0.125% NP-40 (BDH) in PBS) containing 0.12 mM CPRG (chlorophenolred-β-D-galactopyranoside; Calbiochem). After 4 hours incubation at 37°C, 50μl of stop buffer (300 mM glycine and 15 mM EDTA in water) was added to each well and absorption of each well was read using a 96-well plate reader. Absorption wavelength used was 570 nm with 630 nm as the reference wavelength(Karttunen et al., 1992).

2.15 Peptide transport assay

Cells ($3x10^6$) were washed twice with RPMI 1640 media (no serum) and permeabilized for 10 min at 37°C with streptolysin-O (10 units per million cells for K41 and K42 cells; 45 units per million cells for RMA and RMA-S cells). Cells were then washed once with 15ml of buffer (0.005M $MgCl_2$ in PBS) and resuspended in 200μl of warm translocation buffer (78 mM KCl, 4 mM $MgCl_2$, 8.4 mM $CaCl_2$, 10 mM EGTA, 1mM dithiothreitol, 10 mM ATP, 0.1%BSA, 50 mM HEPES, PH7.0). Fluorescein (FITC) tagged RRYQNSTEL peptide described previously(Boname et al., 2004; Neefjes et al., 1993) was added to the cells to a final concentration of 10μM. The cells were incubated at 37°C for 10 minutes, then 1ml of ice-cold lysis buffer (1%NP-40 in PBS) was added and incubated on ice for a further 15 min. Insoluble proteins were removed by centrifugation at 16,000g for 15 min and glycosylated peptides were bound to 50μl concanavalin A sepharose beads (50%(v/v)) by incubating overnight at 4°C with vigorous agitation. The beads were washed three times with 0.1% NP-40 in PBS and bound peptides were eluted with 500μl of 200mmol/L methyl-α-D-mannopyranoside in PBS in the dark at room temperature for 60 min with vigorous agitation. The intensity of fluorescence of supernatant was quantified using a fluorescence spectrometer (PERKIN-ELMER, LS-3) with excitation and emission wavelengths at 484 nm and 535 nm, respectively. The reading was calculated as ratio of fluorescence of transported peptide to the total fluorescent peptide added.

2.16 Real-time PCR for ERp57

RNA was extracted from cells using TRIzol Reagent (Gibco BRL, Gaithersburg, MD) according to the manufacturers' instructions. The integrity of the RNA was confirmed by gel electrophoresis. Reverse transcription was preformed using 5 μg of total RNA, oligo (dT) 1218, and SuperScript II RT (Invitrogen) in a total volume of 20 μl according to the procedure provided by manufacturer. After the reaction, the enzyme was denatured at 70 °C for 15 min. The cDNA was diluted at 1:5 in water. 2 μl of diluted cDNA was used for real time PCR.

Primers for ERp57:

Sense: 5`-GCC TAG GAC TGC TGA TGG AA-3`

Antisense: 5`-CG ATC TCC TTC ACC AGA GAC TCA-3`

GAPDH (used as a control):

Sense; 5'-GAAGGTGAAGGTCGGAGT-3'
Antisense: 5'-GAAGATGGTGATGGGATTTC-3'(Di Como et al., 2002).

PCR reactions were carried out in a volume of 25 μl containing 2 μl of cDNA template, 1.5 μl (10 mM) of each primer, and 12.5 μl SYBR Green PCR master mix (AB Applied Biosystems UK). The products were detected with the ABI 7700 Real-Time PCR Detection System (Bio-Rad) and the cycle values (Ct) were determined as a measure of the cycle number at which a statistically significant increase in fluorescence intensity was first detected and normalized to the value for the control gene GAPDH to yield the relative abundance.

2.17 Immunofluorescence confocal microscopy

K42 and K41 cells transfected with control vector pEF/myc/ER-GFP were trypsinised and replaced on glass cover slides at a density of $0.5\text{-}1\times10^4$ cells/well. Cells were allowed to spread out on the glass slide overnight. The next day, cells were fixed with 4%

formaldehyde for 10 minutes and washed three times in PBS. They were then mounted on slides and cover slips sealed with nail polish before analysis on the confocal microscope.

Chapter 3 Investigation of endogenous antigen processing by delivery of an intact protein in cells

3.1 Introduction

The classic MHC class I antigen processing and presentation pathway uses antigens derived from proteins that are present within the cell. Short peptides are generated by the proteasome and transported via the transporter associated with antigen processing (TAP) into the lumen of endoplasmic reticulum (ER) where peptides can bind to nascent MHC class I molecules(Townsend and Bodmer, 1989). Once MHC class I molecules are properly loaded with antigenic peptide they traffic to the cell surface to interact with T cells(York and Rock, 1996). This pathway plays a crucial role in the generation of cytotoxic T cells (CTL) reactive to cells infected by intracellular pathogens such as viruses and to tumour cells(Brodsky and Guagliardi, 1991). In order to investigate MHC class I antigen processing and presentation, or generation of specific CTL responses against a particular protein, it is therefore necessary to deliver the exogenous antigen into the cytosol so that the protein can be processed inside cells generating epitopes for presentation to T cells.

Several methods have previously been used to deliver antigens into the cytoplasm of cells, including virus infection and plasmid transfection. In both situations the desired antigen protein is expressed inside cells by introducing the encoding gene. However, this relies on introducing the DNA construct rather than protein antigen itself and requires tedious cloning and transfection, or time-consuming procedures for virus production. Furthermore, inconsistent transfection or infection rates for individual experiments make it difficult to quantitatively analyze the efficiency of antigen processing using these methods. Delivery of an accurate amount of protein antigen into the cytosol would be desirable to investigate the efficiency of antigen processing and presentation in a defined system.

There are several methods available for the delivery of protein into the cytosol for the

investigation of protein function, but none of them has gained wide application due to the limitations of each individual method. Microinjection of protein into the cells is an obvious possible method(Narayanan et al., 2003; Abarzua et al., 1995), but the requirement for specialized equipment and sophisticated procedures means that it is only suitable for very small numbers of cells. Electroporation can be used with a variety of cell types, even some cell lines that are resistant to other delivery methods, but the overall efficiency is often quite low and the electroporation process can be damaging to the cells, frequently killing the majority of the cells used (Fenton et al., 1998). Several proteins and small peptides have the ability to transduce or travel through biological membranes, such as the HIV-1 TAT protein and the herpes simplex virus 1 (HSV-1) DNA-binding protein VP22 (Schwarze et al., 2000). A drawback is that the transduction domain must be covalently attached to the protein being delivered, either by creating a DNA construct or by chemical cross-linking. The cationic liposome strategy has also been applied successfully to protein delivery (Zelphati et al., 2001), but properties of an individual protein, such as its charge and hydrophobicity, will influence the efficiency of delivery dramatically. Streptolysin O (SLO) is a family of bacterial exotoxins, which can form pores in the plasma membrance of mammalian cells. Permeablisation appears to be reversible since cells can repair lesions caused by SLO (Bhakdi et al., 1996; Giles et al., 1998). This pore-forming reagent has been used successfully for delivery of anti-sense oligonucleotides into cells (Holasova et al., 2005) and for permitting cellular uptake of molecules for investigation of other cellular processes. Peptides have been delivered by this method for the investigation of TAP peptide transport function (Jun et al., 2000), and whole proteins for investigations of protein function(Fawcett et al., 1998; Walev et al., 2001).

In this chapter, a method using SLO to introduce protein antigen directly into the cells for the investigation of endogenous antigen processing and presentation is described. Cells were treated with SLO and the permeabilized cells then incubated with the desired protein antigen for delivery into the cells. MHC class I antigen processing and presentation of the delivered antigen in these cells was then investigated. The feasibility of this method was demonstrated with a widely used OVA antigen presentation system.

Intact ovalbumin (OVA) was successfully delivered into the cytosol of a mouse fibroblast; antigen was properly processed and the dominant epitope SIINFEKL was generated and presented to B3Z, a unique $CD8^+$ T cell hybridoma restricted to OVA/H-$2K^b$ (Sanderson and Shastri, 1994). By contrast, delivery of OVA protein into RMA-S, a cell line with deficiency of MHC class I antigen processing due to a TAP defect, failed to present the SIINFEKL epitope to B3Z. The results demonstrate an efficient and cost-effective method for delivery of intact protein antigen into cytoplasm for the study of MHC class I antigen processing and presentation. This method permits the efficiency of antigen processing in a defined system to be analysed quantitatively.

3.2 Results

3.2.1 Delivery of intact proteins into the cytosol by streptolysin O

Before investigating endogenous antigen processing by delivery of intact protein, different concentrations of streptolysin O were tested for their ability to permeabalise cells. Under suitable conditions, the cells will be permeablised to allow external protein to enter the cell and allow the cell to repair the lesion caused by streptolysin O treatment so that the cells can be used for further study. To optimise the conditions, a double staining FACS analysis to monitor the efficiency of protein delivery and viability of the cells was adopted. A peptide labelled with FITC was used as an indicator of permeabalisation. Cells loaded with the fluorescein (FITC) tagged RRYQNSTEL would show green fluorescence. After incubation of permeablized cells with labelled peptide, propidium iodide (PI) was used to stain dead cells.

In both mouse fibroblasts and RMA cells, four populations of cells could be seen after treatment with SLO and incubation with FITC tagged peptide (Fig. 3.1): (1) 'unlabelled' - non-permeabilized cells; (2) green only - viable permeabilised cells loaded with peptide; (3) red only - dead cells without peptide loading and (4) red and green - peptide loaded but dead cells. The percentages of these four cell populations varied with increased concentrations of SLO. In the absence of SLO, the bulk of cells exhibited no or very little green or red fluorescence (Fig. 3.1 a,b). With increased dosage of SLO, the proportion of permeabilized cells went up (green cells) at the cost of increased levels of cell death, demonstrated by the PI staining. In RMA cells, when SLO was applied at 30 units per million cells, approximately 32% of the original cell population would remain nonpermeabilized, approximately 14% would be dead, and about 54% would be permeabilized and loaded with FITC labeled peptides. However, when SLO concentration was increased to 60 units per million cells, the nonpermeabilized cell population was slightly reduced to 5%, whereas the percentage of cells in the dead cell

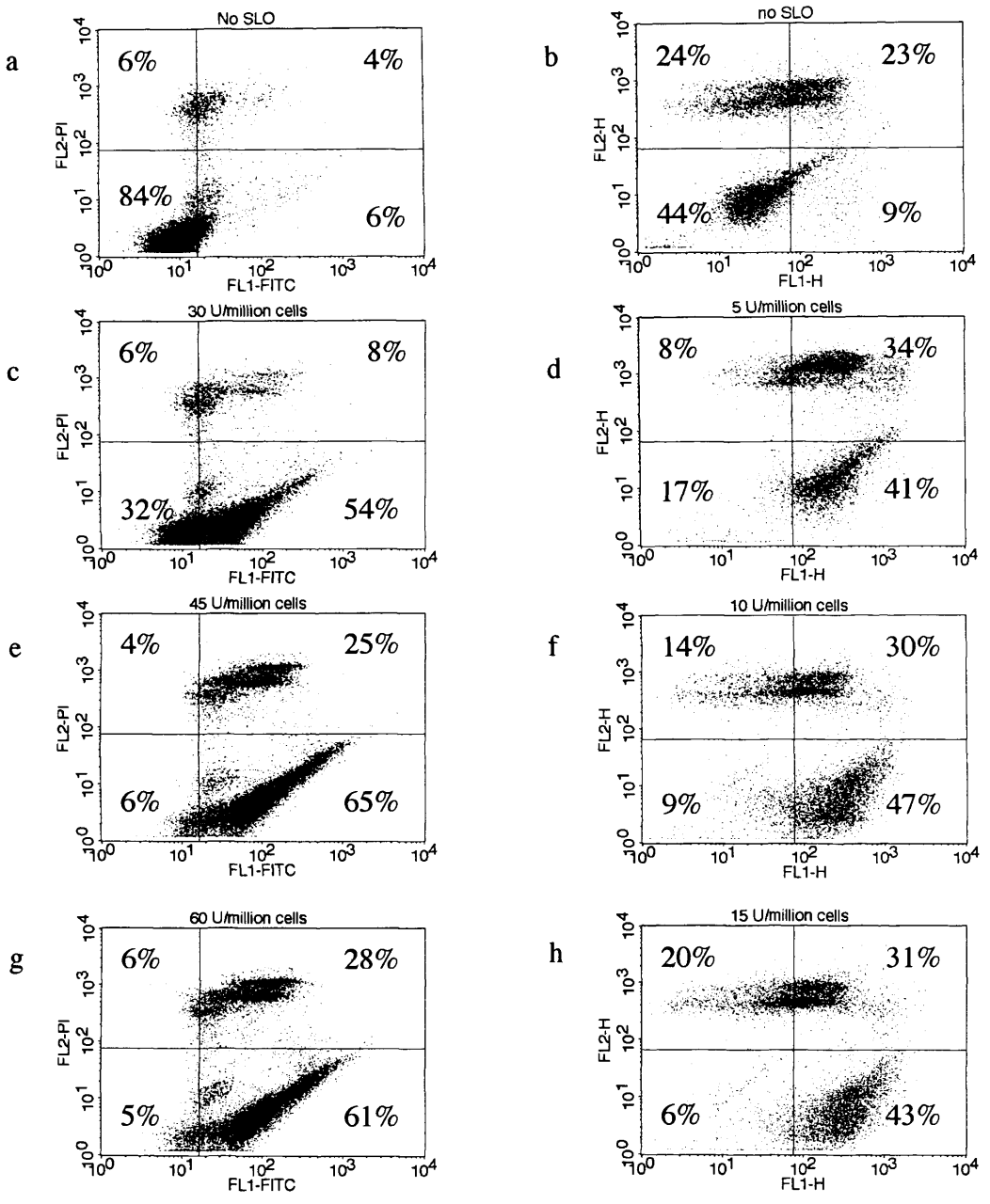


Fig. 3.1 Flow cytometry analysis of cell permeabilization with different concentrations of Streptolysin O

RMA cells (left panel) and Mouse fibroblasts (right panel) were permeabilized using different concentrations of SLO. Cells were incubated with 10μM FITC tagged RRYQNSTEL and subsequently with 20μg/ml PI. After extensive washing, flow cytometry analysis was performed for the presence of FITC and/or PI-labelled cells. Data are representative of three independent experiments.

population went up to 35%, reducing the percentage of live cells loaded with peptide to 61%.

For the mouse fibroblasts used here, 10 units of SLO per million cells was sufficient to obtain a population of 50% of the cells which are live and loaded with FITC labelled peptides in the cytosol (Fig. 3.1). However, for RMA cells a higher concentration of SLO was required to achieve the same efficiency of peptide loading. 45 units of SLO per million cells are used to permeabilize RMA or RMA-S cells to achieve the desired 65% live and permeabalised population (Fig. 3.1 e).

3.2.2 Intact protein delivered by SLO undergoes proper endogenous antigen processing and a dominant epitope was successfully presented to specific T cells.

Once proteins are delivered into the cytosol of cells, they are processed via a conventional MHC class I antigen-processing pathway (Harding and Geuze, 1993). Cytosolic proteins are cleaved by the proteasome, and peptides are transported into the endoplasmic reticulum (ER) through a peptide transporter, TAP. In the ER lumen, epitopes can bind to newly synthesized MHC class I molecules and then traffic to the cell surface to be inspected by cytotoxic T cells (CTL). To find out whether proteins delivered into cytosol by SLO could go through the normal MHC class I antigen-processing pathway, a widely used OVA antigen presentation system (Goldberg et al., 2002) was adopted. This system tests whether intact OVA protein can be properly processed so that the dominant epitope SIINFEKL derived from OVA is presented by mouse MHC class I H-2K^b on the cell surface to B3Z, K^b /SIINFEKL restricted T hybridoma cells. Mouse fibroblast K41 cells were treated with 10 units of SLO per million cells as described in materials and methods. Then Ovalbmin was incubated with permeablised cells to allow antigen to enter the cell. The ability of the OVA derived epitope SIINFEKL to complex with H-2K^b for stimulation of B3Z cells was measured by LacZ activity (Sanderson and Shastri, 1994) following overnight incubation of K41 fibroblasts loaded with OVA with B3Z T cells. As shown in Fig. 3.2, K41 mouse

fibroblasts loaded with OVA by SLO permeabalisation were indeed able to present SIINFEKL epitope derived from cytosolic OVA to B3Z. No activation of B3Z was observed for SLO permeabilized mouse fibroblasts pulsed with a non-relevant control protein BSA (open square), demonstrating that the response of B3Z to the target cells was specific to the OVA derived peptide. The level of B3Z stimulation was close to that stimulated by EG7, an EL4 cell line which endogenously expresses chicken OVA (Moore et al., 1988).

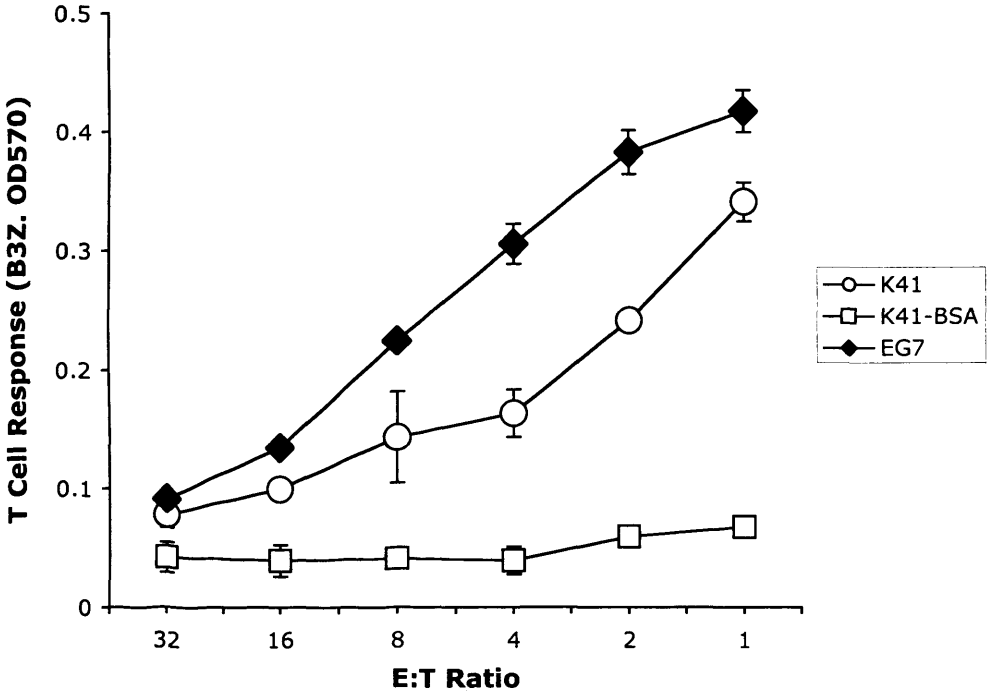


Fig. 3.2 OVA epitope specific T cells can be stimulated by fibroblasts loaded with OVA after treatment with SLO

Mouse fibroblasts permeabilized with SLO were loaded with ovalbumin (OVA) protein or with BSA as a control. Protein loaded cells were co-cultured with the lacZ-inducible, SIINFEKL/K^b-specific B3Z T cell hybridoma at different effector/target (E:T) ratios overnight. The EG7 cell line expressing endogenous OVA was used as a positive control. The B3Z activation level was evaluated by β-Gal activity following overnight incubation as described in the materials and methods. Individual experiments were carried out in triplicate and were repeated at least three times for each cell line.

3.2.3 An endogenous antigen processing defect can be demonstrated in TAP deficient cells loaded with intact protein using SLO

Antigenic peptides generated in the cytosol through the proteasome pathway must, through TAP, enter the ER lumen where peptides are assembled with newly synthesized MHC class I molecules. In the absence of TAP, the peptides generated in the cytosol are unable to be accessed by class I molecules in the ER resulting in a failure of antigen presentation (Kelly et al., 1992; Spies et al., 1992). To find out whether the SLO system can be used to detect endogenous antigen processing defects, OVA was delivered into the TAP-2 locus mutant cell line RMA-S using SLO permeabalisation (Attaya et al., 1992). Due to the known defect for transporting peptides from the cytosol to the ER in this cell line, few peptide loaded MHC class I molecules appear on cell surface, therefore RMA-S cells loaded with OVA using SLO would not be expected to stimulate B3Z cells.

As illustrated in Fig.3.3, when OVA was delivered into the RMA TAP competent parental cell line of RMA, OVA protein was successfully processed and the dominant epitope SINNFEKL was presented in association with H-2K^b on the surface of the cell, as evidenced by efficient stimulation of B3Z. By contrast, in TAP-2 mutant RMA-S cells, there was no activation of B3Z due to the incompetence of translocation of peptides from cytosol to the lumen of ER in these cells. In a control experiment, SLO treated RMA cells pulsed with the irrelevant protein BSA did not stimulate B3Z, demonstrating that the response of B3Z to target cells was specific to OVA peptide.

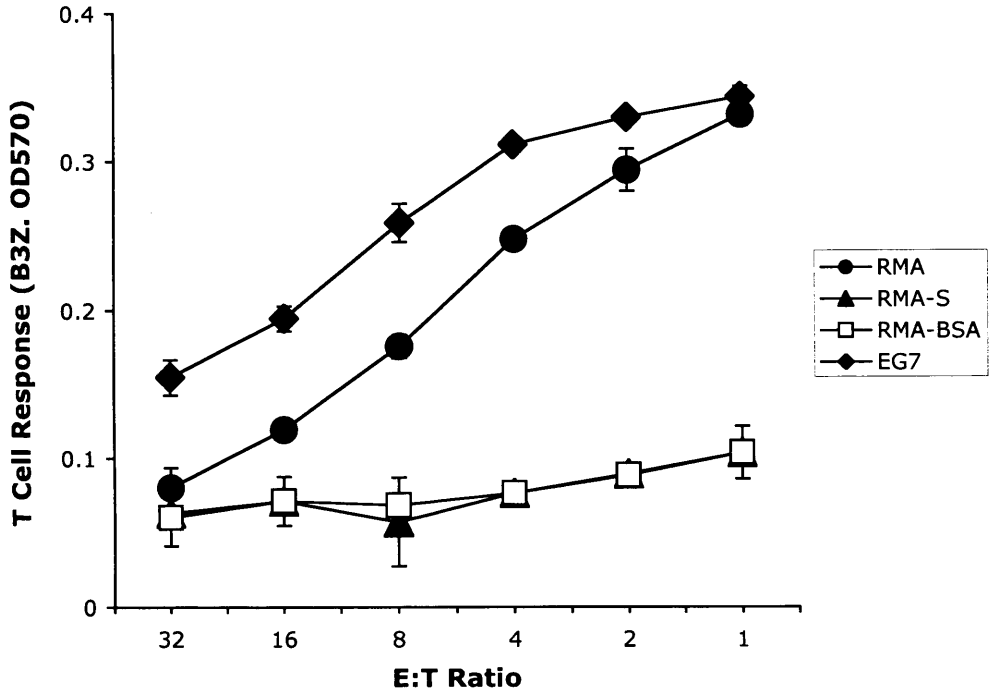


Fig. 3.3 Demonstration of TAP-dependent presentation of OVA derived epitope to T cells by cells loaded with OVA using SLO treatment

Both TAP deficient RMA-S and TAP competent RMA cells were permeabilized using SLO and loaded with ovalbumin (OVA) or with BSA as a negative control. Cells loaded with proteins were co-cultured with B3Z in different effector/target (E:T) ratios. The EG7 cell line expressing endogenous OVA was used as a positive control. The B3Z activation level was evaluated by β-Gal activity following overnight incubation. Experiments were carried out in triplicate and the data shown is a representative experiment of three independent experiments.

3.3 Discussion

To study MHC class I antigen processing and presentation, the antigen of interest must be delivered into the cytosol of cells. Different protocols have been described for this purpose, including microinjection and electroporation of whole protein, and recombinant vaccinia transduction and transfection of cDNA constructs. Electroporation is a widely used in vitro technique that allows direct access of large molecules to the cell cytosol (Song and Harding, 1996). However, electroporation induces cell death in up to 45% of cells by both necrosis and apoptosis due to the damage caused to cell membranes, DNA and chromatin by the electrical charge (Pinero et al., 1997). Microinjection has been used to investigate the role of the ubiquitin-proteasome pathway in generating peptides for presentation on MHC class I molecules (Michalek et al., 1993), but sophisticated equipment and the individual cell based operation restrict the wider application of this method. Transfection of target cells with a plasmid containing a gene of interest, such as Lipofectin or Lipofectamine (Pulaski et al., 1996), requires the construction of intermediate vector expressing desired gene, and the transfected cells need time to express the protein before they can be used for the further study. Furthermore, the transfection efficiency varies between experiments, which makes it difficult to analyze the efficiency of endogenous antigen processing.

Vaccinia infection probably is mostly used system for investigation of MHC class I pathway for a particular protein and can infect a larger fraction of recipient cells (Townsend and Bodmer, 1989). However, vaccinia expression requires construction of vector expressing protein, a time consuming procedure to screen recombined clones and the facility to make recombinant vaccinia viruses. Furthermore, there are several other disadvantages to use the system. (1) the inhibitory effect of vaccinia on epitope presentation to class I-restricted CTL observed during the late phase of vaccinia infection. The possibility is that vaccinia, like other pox viruses, may express one or more serine protease inhibitors that could accumulate in the cytoplasm of infected cells and inhibit host proteases involved in the degradation of antigens recognized by CTL. In

addition the inhibition of host class I molecules during vaccinia infection may contribute to the reduced efficiency of antigen presentation (Townsend and Bodmer, 1989). (2) the dominate epitope derived from vaccina virus itself might corrupt proper epitope processing from the desired protein; (3) non-specific stimulation of T cells. Berwin B et al reported that vaccinia viruses induce necrotic cell death, which yields the release of cellular immunogenic component-GRP94/96 into extra cellular space, which can elicit the activation of OVA-specific hybridomas (Berwin et al., 2001). In our initial experiments, vaccinia viruses expressing OVA were also used to infect mouse fibroblasts to stimulate B3Z T cells for the purpose of investigation of the efficiency of endogenously antigen processed in a defined fibroblast system. However, as shown in Fig. 3.4, vaccinia expressing either irrelevant protein or vaccinia alone, as well as vaccinia expressing OVA were found to be able to stimulate hybridoma B3Z efficiently. So the vaccinia expression system could not be used in our OVA-B3Z model system.

Compared with the above traditional methods, delivery of OVA into the cell cytosol by SLO is efficient, specific, fast and cost-effective. It can achieve a viable cell population with model protein inside the cytoplasm of 50 to 65% of total cells depending on different cell types (Fig. 3.1). Additionally, it is suitable for both adherent and suspended cells. This novel method allows specific stimulation of T cells since target cells pulsed with the irrelevant protein BSA did not activate B3Z, demonstrating that the response of B3Z to target cells is specific to the OVA derived peptide (Fig. 3.2 and Fig. 3.3). The inability of TAP-2 mutant RMA-S cells to activate B3Z demonstrates the failure of endogenous antigen presentation pathway in these cells due to their inability to translocate peptides from cytosol to the lumen of ER (Fig. 3.3). The new technique of delivery of OVA into the cell cytosol by SLO presented here, is quick, 40 minutes is sufficient for the permeabilization target cells and incubation with protein OVA before the co-culture of target cells with effecter cells. Conversely, after transfection or vaccinia infection, a 24 hour cell incubation is needed to allow transfected or infected cells to express the introduced protein. Furthermore, the permeabilization reagent SLO is much cheaper compared to transfection reagents. But the most desirable advantage of using this system is that the efficiency of endogenous antigen processing can be quantitatively

analysed. The protein antigen can be added to SLO permeablized cells in a concentration controlled manner. The intensity of T cell stimulation is proportional to protein antigen delivered into cell (Fig.3.5). Therefore, the efficiency of endogenous antigen processing for a particular protein can be assessed in a defined cell system.

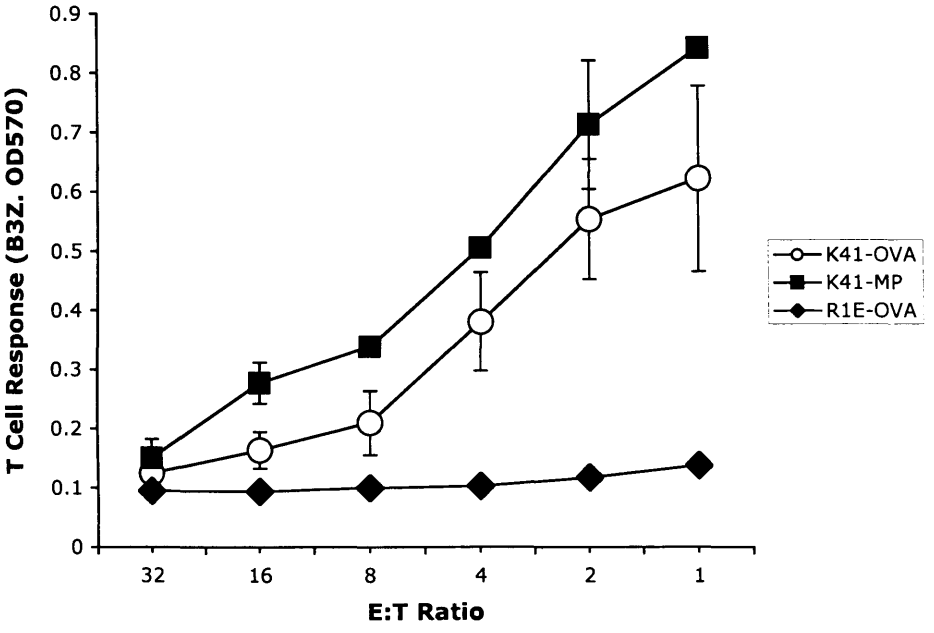


Fig. 3.4 Non-specific stimulation of B3Z by target cells infected with vaccinia virus

Mouse fibroblasts (K41) and β2m deficient R1E cells were infected by vaccinia virus expressing model protein OVA or with vaccinia virus expressing flu matrix protein (MP) as a negative control. Cells were washed, counted, and cocultured with the lacZ-inducible, SIINFEKL/K^b-specific B3Z T cell hybridoma in different effector/target (E:T) ratios. The B3Z activation level after overnight incubation was evaluated as described in materials and methods. The experiments were carried out in triplicate each time and repeated at least three times for each cell line.

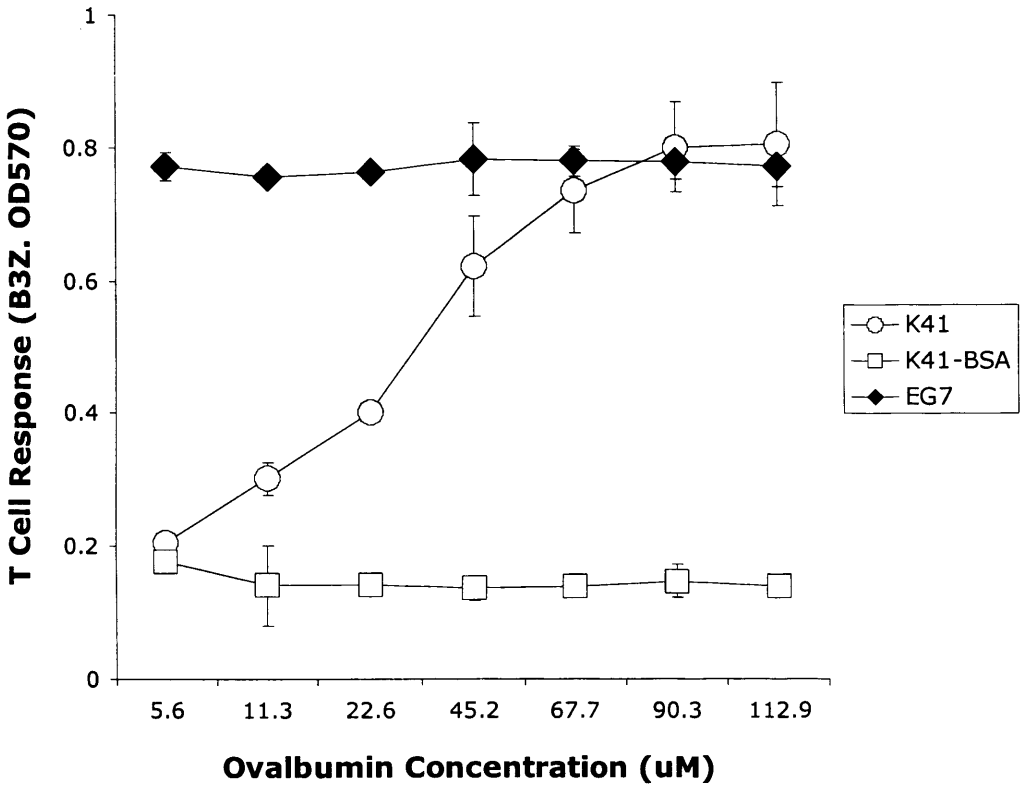


Fig. 3.5 Intensity of T cell stimulation is proportional to protein antigen delivered into cell

Cells were permeabilized using Streptolysin O and different concentrations of Ovalbumin were added to the permeablilized cells. Cells loaded with Ovalbumin were co-cultured overnight with the T cell hybrid (B3Z). T cell activation was measured by colour development in an ELISA as described in the materials and methods. Each experiment was carried out in triplicate for each cell line and repeated at least three times. Data was combined for the plot and error bars show s.d. from the mean.

Chapter 4 Calreticulin maintains the low peptide concentration threshold required in the ER for efficient antigen presentation

4.1 Introduction

Calreticulin (CRT) is an abundant endoplasmic reticulum (ER) resident protein that has a large number of cellular functions in a variety of biological systems. Among these, two of its most important functions are intracellular calcium homeostasis and acting as a chaperone in the folding of newly synthesized glycoproteins(Krause and Michalak, 1997). CRT associates with glycoproteins when they are freshly synthesized and retains them in the ER until they are properly folded. This process of ER retention is an important mechanism in quality control for the production of correctly folded glycoproteins (Hammond and Helenius, 1995; Hebert et al., 1996). CRT has been demonstrated to be involved in class I antigen processing and presentation; it plays an important role in the formation of the peptide-receptive MHC class I complex(Sadasivan et al., 1996) and subsequently in efficient peptide loading(Gao et al., 2002).

Assembly of MHC class I molecules in the ER and their presentation to CD8 T-cells and NK cells is a highly coordinated process involving several critical steps including: the supply of peptides, the generation of peptide receptive class I molecules, the loading of peptide onto class I molecules and ER exit of loaded class I to the cell surface. Peptides are generally derived from newly synthesized polypeptides located in the cytosol where they are cleaved by the proteasome (Cascio et al., 2001; Kloetzel, 2001) and other proteases (Reits et al., 2004; Saric et al., 2004; Stoltze et al., 2000). The position of cleavage generally produces peptides which are extended at the N-terminus but have the correct C-terminus (Cascio et al., 2001). Perhaps assisted by cytoplasmic chaperones (Kunisawa and Shastri, 2003; Dunn et al., 2001), these peptides are transported by TAP into the ER (Powis et al., 1991; Kelly et al., 1992; Androlewicz et al., 1993; Lankat-Buttgereit and Tampe, 1999; Abele and Tampe, 2004) and the extra N-terminal residues

are removed by an aminopeptidase in the ER (ERAAP) (Serwold et al., 2002; Serwold et al., 2001; Saric et al., 2002; York et al., 2002). In the ER, peptides are loaded onto the class I complex through the collaborative work of molecules of the PLC(Cresswell et al., 1999). CRT is closely associated with MHC class I–β2M dimers via its lectin-like binding site and acts as an important member of PLC (Sadasivan et al., 1996). In the absence of CRT, MHC class I molecules are still able to traffic to the cell surface, but the majority of class I molecules are either empty or loaded with sub-optimal peptides, showing that CRT is indispensable for efficient class I assembly and antigen presentation. CRT deficient cell line also fails to present the majority of epitopes tested to specific T cells (Gao et al., 2002). In some respects, the phenotype appears similar to the T134K HC mutant, in which MHC class I molecules exit the ER rapidly as an 'empty' heterodimer. In contrast, empty wild-type MHC class I molecules are normally retained in the ER and degraded. The association of the T134K mutant class I molecules with TAP and CRT is disrupted (Peace-Brewer et al., 1996; Lewis et al., 1996) and this could explain the similarity in the phenotype of the T134K mutant and CRT deficient cells.

As well as being a member of the PLC, CRT has also been shown to bind to peptides transported into the ER by the transporter associated with antigen processing (Spee and Neefjes, 1997). A number of experiments have shown that binding of CRT to peptide may contribute to generating specific immune responses. For instance, immune responses have been generated against tumours by immunising with CRT extracted from them. The immunogenicity is attributed to peptides associated with the extracted CRT and not to the CRT molecule itself (Basu and Srivastava, 1999). It has also been shown that CRT molecules can be complexed in vitro to unglycosylated peptides and used to elicit peptide-specific CD8(+) T cell response by exogenous administration (Basu and Srivastava, 1999). Furthermore, a conjugate of CRT and tumour antigen DNA produced protective immunity against the tumour accompanied with a significant increase in tumour antigen specific CD8(+) T cell precursors (Hsieh et al., 2004; Cheng et al., 2001). Additionally, CRT and other members of the HSP family when associated with peptides can provide a necessary and sufficient source of antigen for cross-priming of CD8 T cells (Binder and Srivastava, 2005).

To further understand how CRT is involved in proper peptide loading onto the class I complex and how it contributes to efficient anti-tumour responses by its adjuvant effect, the involvement of CRT in TAP function, recruitment of β2M to associate with class I HC, and its direct or indirect affect on peptide loading and in the expression and folding of other members of the PLC was investigated.

4.2 Results

4.2.1 TAP deficient phenotype exhibited in CRT knock-out cell line

Previously it has been demonstrated that in a cell line lacking CRT, there is a reduction in MHC class I surface expression compared to a wild type cell line. The reduction is mainly due to the fact that class I is devoid of peptide or loaded with sub-optimal peptide in the ER (Gao et al., 2002). Here evidence is provided that CRT deficient cells have a TAP deficient–like phenotype manifested as unstable class I surface expression, which can be rescued by incubation at lower temperature.

In RMA-S, a murine cell line with a TAP2 defect, class I molecules are formed in the ER but there is a shortage of peptides to load onto them. Empty class I molecules reach the surface of RMA-S cells, but are thermolabile and are degraded at physiological temperature(Ljunggren et al., 1990; De Silva et al., 1999). Although physiological temperature might be optimal for protein function, it might be too high for efficient protein folding and assembly. Consequently, an increase in folding efficiency is often found when cells are grown at reduced temperatures(Hurtley and Helenius, 1989). To assess the stability of class I molecules in the absence of CRT, CRT deficient K42 cells, and their wild type counterparts were incubated at either 26 °C or 37 °C overnight and cell surface class I expression was determined by FACS analysis with anti-class I antibody. As demonstrated in Fig.4.1, when CRT deficient cells K42 were incubated overnight at 37 °C, class I surface expression was reduced about five to seven fold compared to that in wild type K41 cells. However, when K42 cells were incubated overnight at 26 °C, the surface expression of class I of cells was restored to the same level

as in wild type cells (Fig. 4.2). In contrast, there was no difference in class I surface expression between cells cultured at 26 °C and 37 °C in the wild type fibroblasts cell line K41 since the majority of class I in this cell line are properly loaded with peptides (Fig. 4.2). Furthermore, when human CRT (that differs by only a dozen amino acids from its mouse counterpart) was transfected into K42 cells (Fig. 4,3) it restored surface class I expression on K42 to the level of wild type K41 cells (Fig. 4.1). In this case, there was no difference in class I expression between cells incubated at 37 °C and 26 °C (Fig. 4.2). Therefore, the phenotype of K42 cells is similar to that observed in RMA-S, a TAP deficient cell. When RMA-S was cultured at normal physiological temperature (37 °C), there was little class I expressed on the surface of the cell due to quick degradation of empty class I molecules. But when the cells were incubated at 26 °C cell surface expression of class I molecules was restored to normal levels (Fig. 4.2).

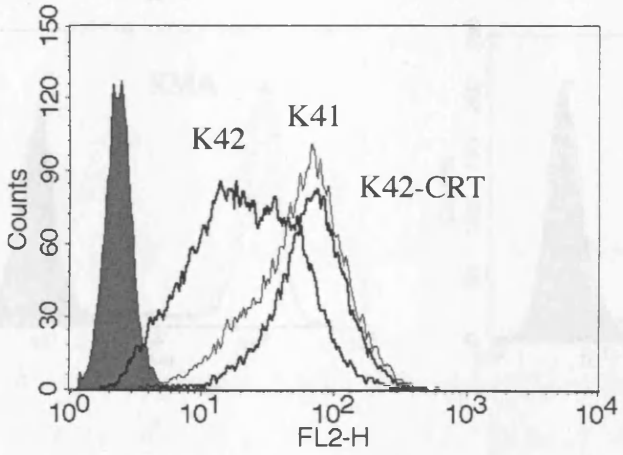


Fig. 4.1 Expression of CRT in K42 cells restored surface MHC class I expression

The surface expression of K^b in K42, CRT K42 transfectant (K42-CRT) and K41 was analysed by FACS staining with the anti-K^b antibody Y3 and rabbit anti-mouse IgG labeled with PE. The shaded peak indicates the staining with secondary PE-conjugated anti-mouse IgG alone.

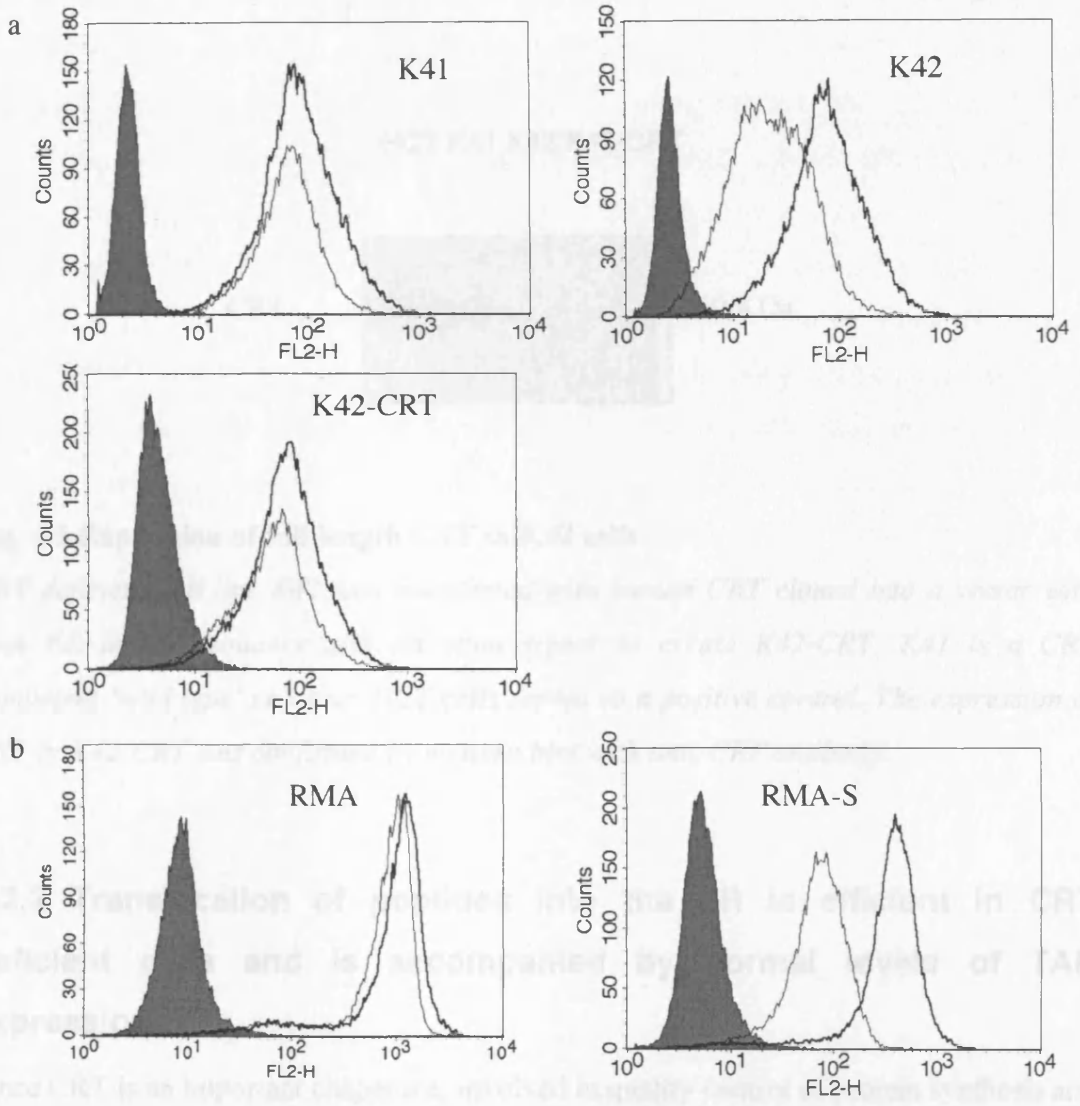


Fig. 4.2 Calreticulin (CRT) deficient cell line exhibits a TAP-deficient phenotype

K42, CRT K42 transfectant (K42-CRT), and CRT competent K41 cells were incubated at either 37°C (thin line) or 26 °C (thick line). MHC class I expression of each cell lines was analysed by FACS staining with the anti-K^b antibody Y3 and rabbit anti-mouse IgG labeled with PE. The shaded peak indicates the staining with secondary PE-conjugated anti-mouse IgG alone. RMA-S, an established TAP deficient cell line, and RMA, the parent cell line of RMA-S with normal TAP function were treated as in (a) and served as a control. All experiments were repeated at least three times. One representative experiment is shown here.

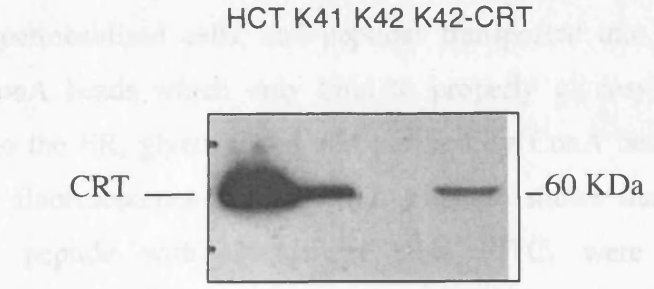


Fig. 4.3 Expression of full length CRT in K42 cells

CRT deficient cell line K42 was transfected with human CRT cloned into a vector with both ER leader sequence and retention signal to create K42-CRT. K41 is a CRT competent 'wild type' cell line. HCT cells served as a positive control. The expression of CRT in K42-CRT was confirmed by western blot with anti-CRT antibody.

4.2.2 Translocation of peptides into the ER is efficient in CRT deficient cells and is accompanied by normal levels of TAP expression

Since CRT is an important chaperone, involved in quality control of protein synthesis and folding and given the fact that the phenotype appears somewhat similar to TAP deficiency, it was important to determine whether there was either a direct or indirect effect on the function of TAP in the CRT deficient cell line. TAP was quantitatively analysed at the protein level by western blot. For protein detection, cells from both the CRT deficient line and their competent counterparts were carefully counted, diluted in a series of two fold dilutions and analysed by western blot using anti-TAP antibody. Level of tapasin expression was used as a loading control since it has been shown previously not to be affected by CRT deficiency (Gao et al., 2002). By western blot there was the same amount of TAP detected in CRT deficient cells compared with CRT wild type cells (Fig. 4.4a). The amount of TAP produced in CRT deficient cells appears to be normal

but this does not prove that its function in the translocation of peptide is unaffected. To clarify this, the efficiency of peptide translocation in CRT deficient cells with was compared that in CRT competent cells. Fluorescent peptide was incubated with Streptlysin O permeablised cells, and peptides transported into the ER by TAP were purified by ConA beads which only bind to properly glycosylated protein. Peptides transported into the ER, glycosylated and purified by ConA beads were quantitatively analysed by a fluoresescence spectrometer. Fig.4.4b shows that an equal amount of RRYQNSTEL peptide with fluorescent label FITC, were transported into the endoplasmic reticulum in CRT deficient cells as in CRT restored cells. This suggests that CRT deficient cells can still efficiently transport peptide into the ER and is consistent with the normal level of TAP in these cells. As a control, TAP-2 mutant RMA-S cells were demonstrated to fail to transport the FITC labelled RRYQNSTEL across the ER membrane, while TAP competent RMA cells were capable to do so.

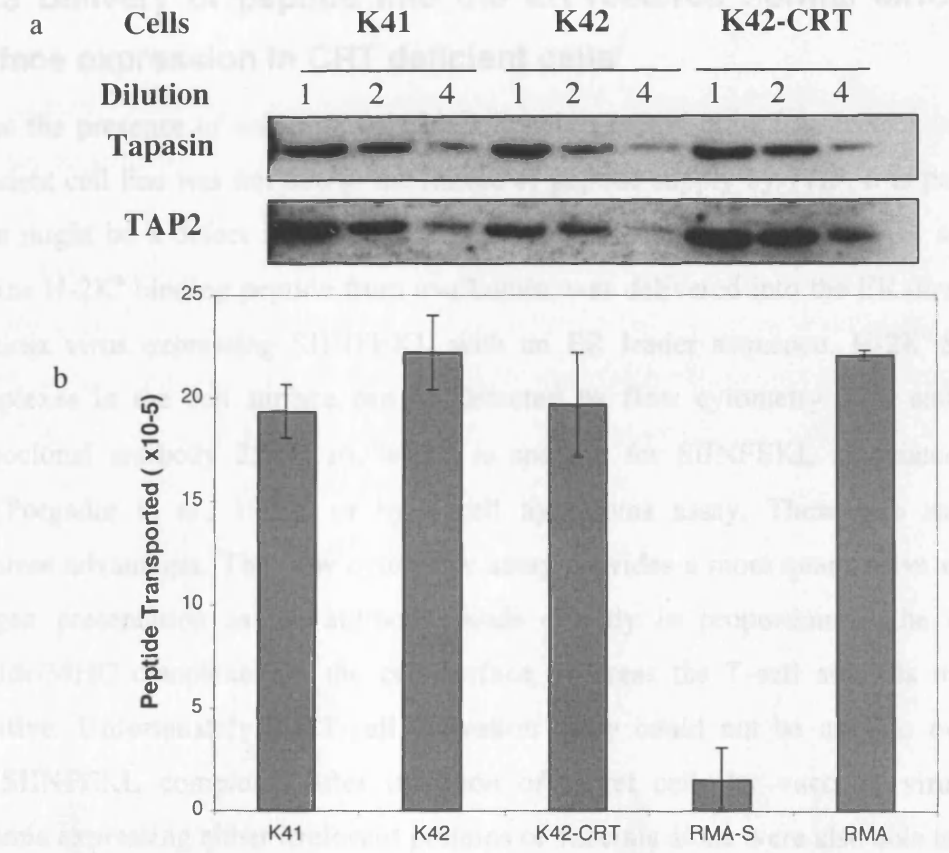


Fig. 4.4 TAP expression and peptide transportation efficiency in CRT deficient cell line K42 compared to that in CRT competent cell line K41

a: Cells were counted and lysed with 0.5% NP-40 in PBS. Two fold diluted lysates were run on 12%SDS-PAGE and the proteins were transferred onto cellulose membrane, which was then probed with anti-tapasin and anti-TAP2 rabbit serum.

b: cells permeablised with streptolysin O and then incubated for ten min in translocation buffer containing FITC labeled RRYQNSTEL to test their capacity to transport peptides into the ER. Transported peptides having undergone N-linked glycosylation in ER were recovered by precipitation with Con A-sepharose from lysed cells. The fluorescent intensity of recovered FITC labelled RRYQNSTEL was quantified using a fluorescence spectrometer as described in the material and methods. Data are presented as the percentage of the total peptides that were transported and recovered from lysates and shown as means ±SD of three independent experiments.

4.2.3 Delivery of peptide into the ER restored normal MHC class I surface expression in CRT deficient cells

Since the presence of sub-optimally loaded and/or empty class I molecules in the CRT deficient cell line was not due to the failure of peptide supply by TAP, it is possible that there might be a defect in peptide loading itself. To test this, SIINFEKL, a dominant murine H-2K^b binding peptide from ovalbumin, was delivered into the ER directly using vaccinia virus expressing SIINFEKL with an ER leader sequence. H-2K^b:SIINFEKL complexes in the cell surface can be detected by flow cytometry after staining with monoclonal antibody 25-D1.16, which is specific for SIINFEKL associated with H-2K^b(Porgador et al., 1997), or by T-cell hybridoma assay. These two assays have different advantages. The flow cytometry assay provides a more quantitative measure of antigen presentation as the antibody binds directly in proportion to the density of peptide/MHC complexes on the cell surface, whereas the T-cell assay is much more sensitive. Unfortunately the T-cell activation assay could not be used to evaluate H-2K^b:SIINFEKL complexes after infection of target cells by vaccinia virus because vaccinia expressing either irrelevant proteins or vaccinia alone were also able to stimulate the B3Z hybridoma (Fig 3.4). Vaccinia viruses can induce necrotic cell death, which causes the release of the cellular component-GRP94/96 into the extra cellular space and elicits B3Z activation(Berwin et al., 2001). Therefore, the expression of SIINFEKL loaded K^b was analysed using the mAb 25-D1.16.

First vaccinia titration assays were done on normal mouse fibroblasts. In Fig.4.5, by staining with mAb 25-D1.16 and analysing by FACS, it is shown that, when different amounts of SIINFEKL were delivered into normal murine fibroblast cells the number of cells expressing K^b/SIINFEKL is proportional to the multiplicity of infection (MOI) of vaccinia used. While staining with mAb Y3, an antibody recognising conformation-dependent H-2K^b bound to any peptide, showed that little change was observed in total K^b expression between cells infected by different amount of vaccinia virus. 25-D1.16 staining can be used to measure the fraction of K^b molecules loaded with SIINFEKL at the cell surface. The result of vaccinia virus titration assay revealed that when more vaccinia virus

was used to infect cells, a higher percentage of cells was infected to express SIINFEKL peptides, but the less viable cells were left expressing SIINFEKL peptides because vaccinia virus caused cell death(Berwin et al., 2001). Therefore, 25µl of the same batch of vaccinia virus was used to infect the same number of cells and evaluate the ability of different cell lines to load the SIINFEKL peptide onto class I H-2K^b molecules and express them at the cell surface. The data shown in Fig.4.6 demonstrated that the surface expression of class I molecules on CRT deficient K42 could be restored to the same level as that in CRT competent cell lines by delivering SIINFEKL peptide directly into ER. This suggests that the loading of peptide onto class I molecules is normal in CRT deficient cells as long as enough peptide is delivered into the ER.

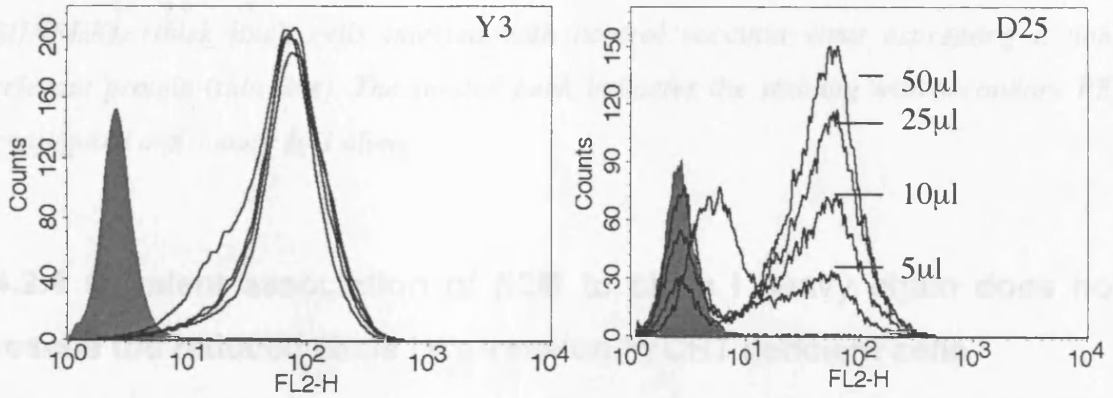


Fig. 4.5 Titration of vaccinia virus

Murine K41 fibroblast cells were infected with different amount of vaccinia virus expressing the ER leader sequence followed by SIINFEKL. Expression of total murine MHC class I K^b and of K^b/SIINFEKL on the surface of cells was analysed by FACS using the monoclonal antibodies Y3 (left panel) and 25-D1.16 (right panel). The shaded peak indicates the staining with secondary PE-conjugated anti-mouse IgG alone.

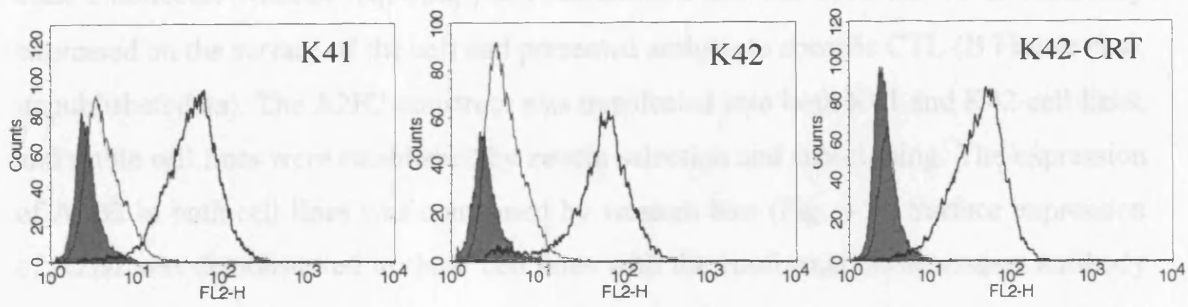


Fig. 4.6 MHC class I surface expression was restored when SIINFEKL was directly delivered into the ER

K41, K42 and K42-CRT were infected with 25 μl of vaccinia expressing ER targeted SIINFEKEL, and surface expression K^b/SIINFEKL was analyzed by flow cytometry using 25-D1.16 antibody. Histograms show cells infected with vaccinia virus expressing SIINFEKL (thick line), cells infected with control vaccinia virus expressing a non-relevant protein (thin line). The shaded peak indicates the staining with secondary PE-conjugated anti-mouse IgG alone.

4.2.4 Covalent association of β2M to class I heavy chain does not rescue the reduced class I expression in CRT deficient cells

It is widely accepted that when class I HC is newly synthesized it associates with calnexin (Nossner and Parham, 1995; David et al., 1993) to maintain a β2M receptive state. β2M is then recruited to associate with class I HC and this is accompanied by the replacement of calnexin with CRT (Sadasivan et al., 1996). Although there is no direct evidence to show how CRT is involved in the replacement of calnexin and in bringing beta-2-microglobulin into proximity with HC, given the order of protein interactions it is possible that CRT may be responsible either for promoting the assembly of class I HC with β2M or for the recruitment of β2M.

To investigate a possible defect in the recruitment of β2M to class I HC in the CRT deficient cell line, a fusion protein of β2M was covalently linked to HLA-A2 (A2B2) was transfected into K41 and K42 cells by B Flutter. This fusion protein should fold into a

class I molecule without requiring β2M recruitment and has been shown to efficiently expressed on the surface of the cell and presented antigen to specific CTL (B Flutter et al: unpublished data). The A2B2 construct was transfected into both K41 and K42 cell lines, and stable cell lines were established by zeocin selection and sub-cloning. The expression of A2B2 in both cell lines was confirmed by western blot (Fig. 4.7). Surface expression of A2B2 was demonstrated in these cell lines with the conformation-dependent antibody W6/32, suggesting that linked chain was folded properly in both cell lines and was able to traffic normally to the surface of the cell. Surface expression of the A2B2 in the CRT-deficient cell line was significantly lower than that on the CRT competent cell line (Fig. 4.8). The observed difference in linked chain expression was similar to the difference in murine class I surface expression between CRT-deficient and competent cells. Since pre-assembled A2B2 does not overcome the calreticulin deficiency in K42 cells, the recruitment of β2M to class I is unlikely to be the reason for the class I assembly defect in the absence of CRT.

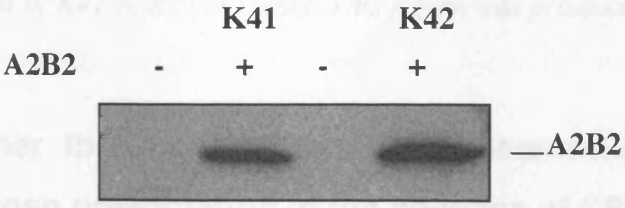


Fig. 4.7 Expression of linked chain HLA-A2- β2M (A2B2) in both K42 and K41 cells.

Expression of fusion protein A2B2 was confirmed by western blot using the antibody BBM1, specific for human β2M. This figure was produced by Dr Barry Flutter.

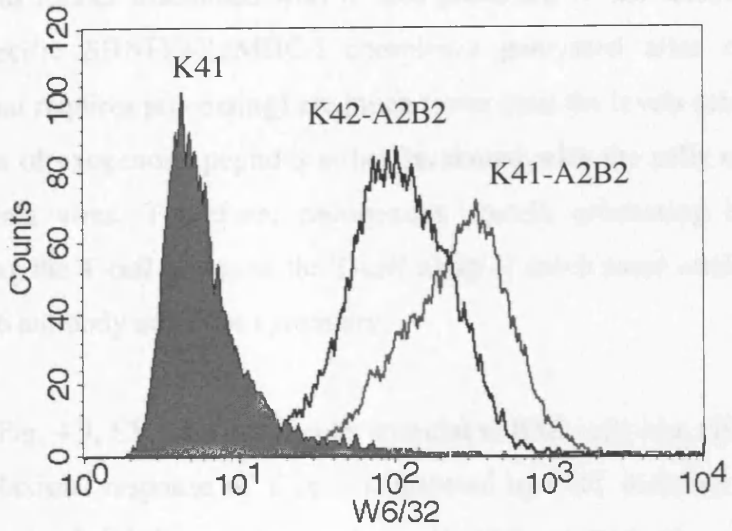


Fig. 4.8 Class I HC pre-linked to beta-2-microglobulin failed to restore normal class I expression in CRT deficient cells.

Cells were stained with W6.32 antibody against human class I and analysed by flow cytometry. K42 cells transfected with A2B2 expressed lower levels of human class I compared to that of K41 (CRT+/+) cells. This figure was produced by Dr Barry Flutter.

4.2.5 A higher threshold of peptide concentration is required for efficient antigen presentation in the absence of CRT

Since peptide loading onto MHC class I molecules in ER was not impaired and TAP function was normal in calretciulin deficient cells, it was thought that CRT might play a role in the maintenance of the low threshold of peptide required in the ER for efficient presentation to T cells. This might occur through the enrichment of the peptide in the ER or by promoting efficient assembly of the PLC. To test this idea, the activation of a specific T cell by antigen presenting cells loaded with controlled concentrations of whole protein antigen was investigated. To achieve a controlled protein loading into the cell, the new method developed in chapter 3 using streptolysin O to temporarily permeablise the cells was used. Ovalbumin (OVA) antigen was added to the permeabilised cells and loaded into the cytosol of the cell. OVA was processed inside the cell and the peptides degraded from OVA transported into the ER. In the ER, the dominant peptide

SIINFEKL was further assembled with K^b and presented on the surface of the cell. The levels of specific SIINFEKL:MHC-I complexes generated after exposing cells to ovalbumin (that requires processing) are much lower than the levels achieved with higher concentrations of exogenous peptides either incubated with the cells or delivered to the ER by vaccinia virus. Therefore, endogenous protein processing experiments were evaluated using the T-cell assay, as the T-cell assay is much more sensitive than staining with 25-D1.16 antibody and flow cytometry.

As shown in Fig. 4.9, CRT deficient cells stimulated B3Z cells less effectively than wild type cells. Maximal response of T cells stimulated by CRT deficient cells pulsed with optimal amount of OVA was lowered significantly compared to wild type cells. Detectable stimulation required about a 3-fold higher ovalbumin concentration in CRT deficient cells than wild type cells, suggesting that the concentration of ovalbumin required for efficient antigen presentation is lowered in the presence of CRT. Furthermore the increased threshold of OVA antigen required for antigen presentation in CRT deficient cells could be corrected by transfecting CRT back into the deficient cells (K42-CRT). Noticeably, in CRT transfected cells, the stimulation of B3Z was stronger than in wild type cells. Since transfected cells express a high level of CRT, this further supports the notion that CRT may promote efficient antigen processing. In control experiments, both streptolysin O treated cells pulsed with non-relavent protein BSA and untreated cells incubated with OVA failed to stimulate B3Z, demonstrating that the response of B3Z to the target cells treated with streptolysin O and pulsed with OVA is specific (Fig. 4.9).

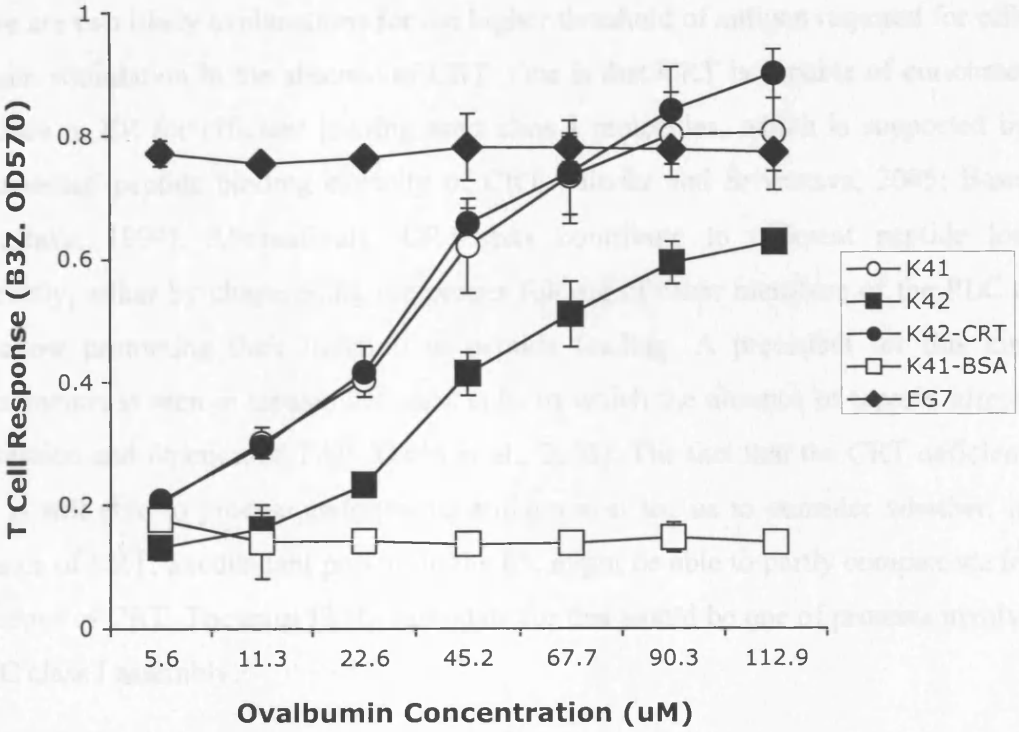


Fig. 4.9 CRT maintains a low threshold of peptides required in the ER for efficient antigen presentation to T cells

Cells of K41, K42, CRT K42 transfectant (K42-CRT) were permeabilized using Streptolysin O and different concentrations of Ovalbumin with BSA as a control were added to the permeablilized cells. Cells loaded with Ovalbumin were then co-cultured overnight with the T cell hybrid (B3Z). T cell activation was measured by colour development in an ELISA as described in the materials and methods. EG7 was used here as experimental control. Each experiment was carried out in triplicate for each cell line and repeated at least three times. Data was combined for the plot and error bars show s.d. from the mean.

4.2.6 Up-regulation of ERp57, but normal level of tapasin and calnexin expression in CRT deficient cells

There are two likely explanations for the higher threshold of antigen required for efficient antigen stimulation in the absence of CRT. One is that CRT is capable of enrichment of peptides in ER for efficient loading onto class I molecules, which is supported by the documented peptide binding capacity of CRT (Binder and Srivastava, 2005; Basu and Srivastava, 1999). Alternatively, CRT may contribute to efficient peptide loading indirectly, either by chaperoning the proper folding of other members of the PLC or by somehow promoting their function in peptide loading. A precedent for this kind of phenomenon is seen in tapasin deficient cells in which the absence of tapasin affects the expression and function of TAP (Garbi et al., 2003). The fact that the CRT deficient cell line is still able to process endogenous antigen also led us to consider whether, in the absence of CRT, a redundant protein in the ER might be able to partly compensate for the functions of CRT. The most likely candidate for this would be one of proteins involved in MHC class I assembly.

To find out if any other member of the PLC is affected by the absence of CRT, individual components of the PLC were quantitatively analysed by western blot. Cells were counted, lysed in 1% NP40, serially diluted in PBS and analysed by western blot with antibodies to calnexin, tapasin, HC and ERp57. Accurate cell counting between samples was again confirmed by equal calnexin expression. In contrast to other components of the PLC, the expression of ERp57 in K42 cells was increased at least two fold compared to that in both CRT transfected K42 and competent wild type cells (Fig. 4.10). This increased level of protein expression is consistent with an upregulated level of mRNA transcription confirmed by quantitative real time PCR (Fig. 4.11). As with calnexin and TAP, there was no significant difference in the expression of tapasin between CRT deficient and competent cells (Fig. 4.10). However, under carefully controlled conditions it was shown that there were more class I HCs accumulated in CRT deficient cells than that in either CRT wild type cells or CRT deficient cells transfected with CRT (Fig. 4.10). This is not consistent with previous observations (Gao et al., 2002). However, the level of total HC in CRT deficient cell lines detected by western blot is very sensitive to minor differences

in experimental procedure. For example, the length of time cells are digested by trypsin and even the amount of time the cells are kept in suspension before lysis could affect protein level (unpublished observation). In this report all assay conditions were made as consistent as possible.

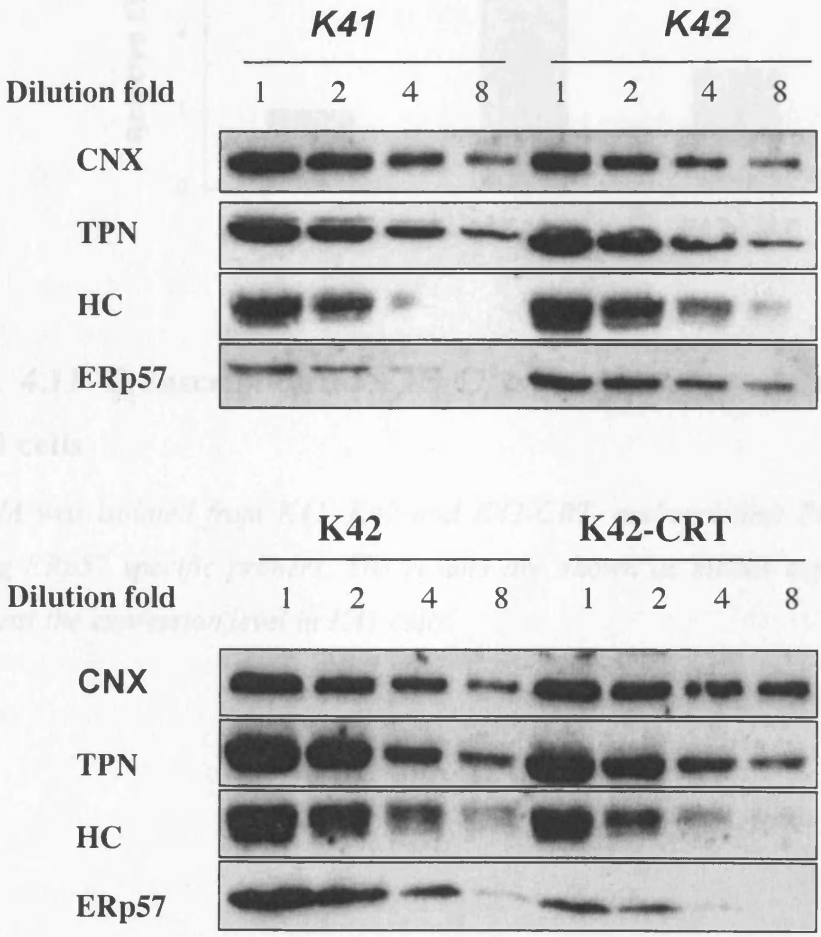


Fig. 4.10 Up-regulation of ERp57 but normal expression of tapasin, TAP and calnexin in CRT deficient cells

Two-fold dilutions were made on cell lysates of K41, K42 and K42-CRT, and samples were separated on 12% SDS-PAGE. Individual proteins were detected by western blot using the antiserum to calnexin (CNX), tapasin (TPN), HC and ERp57.

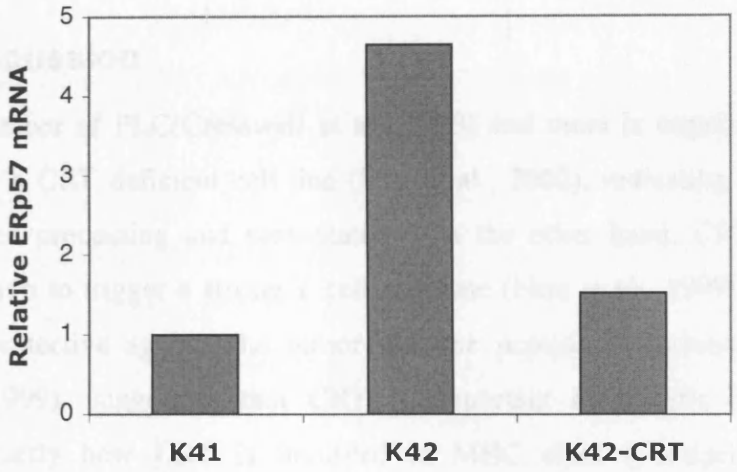


Fig. 4.11 Transcription of ERp57 was up-regulated in CRT deficient K42 cells

mRNA was isolated from K41, K42 and K42-CRT, and real time PCR was carried out using ERp57 specific primers. The results are shown as mRNA expression normalized against the expression level in K41 cells.

4.3 Discussion

CRT is a member of PLC(Cresswell et al., 1999) and there is impaired class I surface expression in a CRT deficient cell line (Gao et al., 2002), indicating its critical role in class I antigen processing and presentation. On the other hand, CRT associates with peptides in vivo to trigger a strong T cell response (Nair et al., 1999) and the response initiated is protective against the tumor that the peptide is derived from (Basu and Srivastava, 1999), suggesting that CRT is important in specific T cell responses. However, exactly how CRT is involved in MHC class I antigen processing and presentation and how it contributes to anti-tumor responses remains a mystery. Using an OVA protein based antigen processing assay it has been shown here that significantly higher antigen concentration is required for efficient presentation of the dominant epitope SIINFEKL to specific T cells in CRT deficient cells compared to that in normal cells (Fig. 4.9). Therefore, CRT reduces the ER peptide concentration needed for efficient MHC class I antigen presentation to T cells. The impaired endogenous antigen presentation in CRT knockout cells was also mentioned in the previous work done by Gao et al(Gao et al., 2002). They tested the ability of CRT deficient cells (K42) to present the K^b–restricted OVA-derived epitope SIINFEKL following infection with vaccinia virus expressing cytosolic OVA and showed that at all doses of virus used to infect cells, between 2- to 3-fold more SIINFEKL: K^b complexes, detected by mAb 25-D.16, were found on the surface of CRT competent cells than CRT deficient cells(Gao et al., 2002). The function of CRT in lowering the peptide concentration threshold required in the ER for class I antigen presentation has physiological importance, especially in virus-infected cells. Studies have demonstrated that viruses have evolved remarkable mechanisms to inhibit MHC class I expression by interfering with the function of the MHC class I assembly machinery to evade the immune response. An efficient way to inhibit class I expression is to cut off the source of peptides(Lybarger et al., 2005). For example, herpes simplex virus-infected cells express the viral evasion protein ICP47, which binds to the TAP peptide transporter and blocks peptide translocation into the ER(Ahn et al., 1996). By doing so, herpes simplex virus down regulates the surface class I expression to avoid

recognition by CTLs. By reducing the peptide threshold in the ER, CRT is likely to be important so that virus-infected cells could present virally derived antigenic peptides efficiently to CTLs when they are limited in the lumen of ER.

In this chapter, the possible mechanisms by which CRT reduces the ER peptide concentration needed for efficient MHC class I antigen presentation to T cells were investigated. It was shown that CRT deficient cells exhibited a TAP deficient phenotype in terms of class I assembly. Normal surface expression of MHC class I molecules in CRT deficient cells could be restored by incubation of cells at 26 °C. At this low temperature, empty or sub-optimal peptide loaded class I were more stable than that at 37 °C. Based on the previous experimental observation that correlates the thermostability of a MHC class I complex with the affinity of its peptide cargo(Williams et al., 2002b), the data suggested that the MHC class I molecules in CRT deficient cells were peptide-receptive (empty) or suboptimally loaded. This result was consistent with previous work showing that the ability of intracellular loading of K^b with peptides in K42 by immunoprecipitating a cohort of newly synthesized class I molecules in the presence or absence of peptide ligand using either conformation-sensitive mAb (Y3) or an antiserum (T18) which only recognizes the β2m free K^b and found that a greater proportion of class I molecules in CRT deficient cells were peptide receptive(Gao et al., 2002).

There are three possibilities why empty or suboptimally loaded MHC class I molecules appear on the cell surface of K42: shortage of peptide supply, impaired quality control and/or peptide-loading defect in the ER.

These possibilities were investigated. Experiments were first carried out to determine whether there is shortage of peptides in CRT deficient cells. TAP is responsible for transporting peptides from cytosol to the ER. In TAP deficient cells, most class I molecules fail to assemble with stably bound peptide duo to the shortage of peptide supply and are therefore retained in the ER(Grandea, III et al., 2000; Schoenhals et al., 1999). These empty class I molecules that accumulate in the ER are eventually translocated to the cytoplasm for degradation(Van Kaer et al., 1992; Wiertz et al., 1996).

Consequently, they are unable to present intracellular antigens on the cell surface(Hosken and Bevan, 1990; Spies et al., 1992). However, the peptide translocation assay and western blot did not provide any evidence of abnormality in the expression and function of TAP in CRT deficient cells (Fig. 4.4), which suggested that peptide supply from cytosol to the ER was unchanged in the absence of CRT. Taking all these findings into consideration, it is hypothesized that CRT plays a critical role in the enrichment of peptides in the ER for loading onto class I molecules, therefore maintaining a low peptide concentration threshold for efficient antigen presentation. If CRT maintains a lower peptide concentration threshold for efficient antigen presentation, it is not surprising that while some epitopes cannot be presented by CRT deficient cells certain epitopes were still able to be properly processed and presented to corresponding T cells (Gao et al., 2002). These presented epitopes could simply be more abundant or have a sufficiently high affinity for class I molecules so that they do not require enrichment by CRT. The efficiency of generation of a specific class I/peptide complex from a given antigen differs enormously between different antigens. It has been demonstrated that Listeria monocytogenes-infected macrophages generate about 10 class I/peptide complexes per 100 lesteriolysin O proteins degraded, and three class I/peptide complexes per 100 p60 proteins degraded(Villanueva et al., 1994). It is possible therefore that a higher efficiency in the number of class I/peptide complexes per degraded antigenic protein may result in a lesser dependency on CRT for enriching peptide concentration in the ER. Furthermore, the result that CRT maintains a lower peptide concentration threshold for efficient antigen presentation also provides a possible logical explanation as to why CRT could be used with peptides to invoke efficient anti-tumor immunity.

The involvement of CRT in the formation of peptide-receptive class I complex is well documented. When class I molecules are newly synthesised they associate with calnexin (Nossner and Parham, 1995; Degen and Williams, 1991; David et al., 1993), which in humans binds only to free HC(Rajagopalan and Brenner, 1994). Subsequently when HC binds β2M, CRT replaces calnexin. Mouse HCs can bind simultaneously to both calnexin and CRT whether expressed in human or mouse cells, reflecting extra glycosylation sites on mouse HC (in addition to position 86, mouse HCs are also glycosylated at Asn176 for

K^b and Asn176/Asn256 for D^b (Evans et al., 1982; Maloy and Coligan, 1982; Nathenson et al., 1981; Helenius and Aebi, 2001). In mouse cells calnexin binds preferentially to free HC in contrast with the association of CRT with MHC class I molecules which requires HC assembly with β2m (Nossner and Parham, 1995).Considering that CRT is not associated with HCs in a β2M deficient cell line (Sadasivan et al., 1996) and calnexin was found not to co-exist with properly folded class I molecules in human cells (Rajagopalan and Brenner, 1994), it has been suggested that CRT and β2M might replace calnexin to produce a peptide-receptive class I heterodimer. CRT might be required for β2M recruitment, and reduced surface class I expression in CRT deficient cell lines might be related to the failure in β2M recruitment. However, a pre-tethered class I β2M–HC dimer was unable to rescue impaired class I expression in CRT deficient cells, clearly demonstrating that CRT is not essential for the recruitment of β2M to associate with HC (Fig. 4.8).

Using semiquantitative western blot, the steady-state protein expression level of HCs in CRT deficient cells was found to be higher than that in CRT competent cells (Fig. 4.10). Although experimental conditions could affect the level of total HCs in CRT deficient cell lines detected by western blot, in this study all assay conditions were kept as consistent as possible and the amount of protein loaded was strictly controlled. MHC class I HC is a glycoprotein with two highly conserved disulfide bonds (α2 and α3 domains) (Oliver et al., 1999). It associates early during biosynthesis with calnexin(David et al., 1993; Degen and Williams, 1991; Degen et al., 1992) and with ERp57(Lindquist et al., 1998; Lindquist et al., 2001) to facilitate the folding of HC and stabilize it from aggregation as well as promote its assembly with β2m. ERp57 has been proposed to function as a 'co-chaperone' of CNX to modulate disulfide-bond isomerization of class I molecules and promote class I HC folding(Farmery et al., 2000; Molinari and Helenius, 1999; High et al., 2000; Ellgaard and Helenius, 2001). It is possible, therefore, that the higher level of ERp57 in CRT deficient cells increases the folding efficiency of class I HC, which results in the accumulated class I HCs in cells. Additionally, Gao and colleagues have demonstrated that the total amount of K^b assembled with β2m was

similar in CRT deficient K42 and CRT competent cells, and the peptide-receptive class I molecules released to the cell surface of K42 had the same affinity for the peptide ligand as they did in K41(Gao et al., 2002). Taken together, these results indicated that the empty or suboptimally loaded MHC class I molecules on the cell surface of CRT deficient cells were not related to the formation of peptide receptive class I heterodimer or its affinity for the peptide ligand.

CRT is an important molecular chaperone for glycoproteins in the ER. CRT together with calnexin and ERp57, constitute the "CRT/CNX cycle", which is responsible for the folding, oligomeric assembly and quality control of newly synthesized glycoproteins, and helps to retain in the ER misfolded or incompletely assembled proteins, which are then targeted to the cytosol for degradation(Peterson et al., 1995; Wada et al., 1995). This quality control system works at a general level that is applied to nearly all proteins. Therefore, CRT is expected to take part in proper folding of members of PLC, such as calnexin, tapasin, TAP and ERp57. To assess the effect of loss of CRT on the presence and amount of these ER-localized chaperones and folding enzymes, semiquantitative western blot was used to study the steady-state protein expression level of these proteins. In these experiments, the amount of TAP (Fig. 4.4a), tapasin or calnexin expressed was not affected by the absence of CRT (Fig. 4.10). Although it is not possible to test the functionality of all these proteins translocation of peptides by TAP was normal in the absence of CRT (Fig. 4.4). Interestingly, there was about a 2-4 fold increase in the level of ERp57 in CRT deficient cells shown by western blot (Fig. 4.10) and confirmed by real time PCR (Fig. 4.11). It is well documented that CRT interacts with ERp57 to contribute to class I folding, for example, CRT forms one to one complexes with ERp57 to modulate MHC class I folding (Oliver et al., 1997). The tip of the arm-like P-domain of CRT interacts with ERp57 allowing it to interact optimally with cysteines in the class I HC and catalyse the formation of two intra-chain disulfide bonds in MHC class I HC (α2 and α3 domains) (Oliver et al., 1999). Stable association of MHC class I-heterodimers with CRT appears to be a prerequisite for their association with TAP via tapasin (Sadasivan et al., 1996), and although some tapasin can bind to class I molecules in the absence of CRT, the majority of tapasin is unable to associate with class I molecules (Sadasivan et al.,

1996). Since CRT, not calnexin, shows predominant association with the TAP complex, CRT could aid the stabilization and/or the ER retention of β2m-assembled H chains awaiting peptide(Harris et al., 1998). Indeed, since CRT binds to newly formed HC-β2M heterodimers, ERp57 and the TAP complex, it has been suggested that the binding of CRT to class I is likely to induce specific effects on the folding of polypeptide chain, which may help in mediating the association of class I with tapasin/TAP and ERp57 complex(Sadasivan et al., 1996). CRT may act as an initial bridge between peptide free class I molecules and the PLC.

It is worth mentioning that Helenius and colleagues concluded that there was no significant increased ERp57 expression in CRT deficient mouse embryonic fibroblasts based on western blotting results(Molinari et al., 2004). However, the western blots shown in their study appeared to have an increase in the expression of ERp57 to at least the same level as BiP highlighted by the authors. Furthermore, enhanced ERp57 expression might explain the observation that hemagglutinin was fully oxidized and six intramolecuelar disulfide bonds were formed more efficiently in CRT deficient cells than that in normal cells(Molinari et al., 2004). Addtionally, proteomic analysis of C. Elegans also indicated that there was a dramatic increase in expression of ERp57 in CRT deficient worms compared to that in wild type counterparts(Lee et al., 2006). ERp57 interacts with CRT and calnexin to assist in folding of N-glycosylated substrates(Oliver et al., 1997; Zapun et al., 1998). Perhaps the association of ERp57 with CRT is important for the maturation of class I molecules, but whether the elevation of ERp57 is a compensative measure for the defect in CRT deficient cells is not clear at this time. Both CRT and ERp57 have been demonstrated to maintain the integrity of the PLC. For example, in the absence of CRT, less MHC class I molecules were present in the PLC(Gao et al., 2002). Similarly, Garbi and colleagues have found that very few class I molecules were present in the loading complex of ERp57 deficient cells(Garbi et al., 2006). ERp57 is involved at different steps of the MHC class I pathway, first during biosynthesis and later as a component of the PLC. Less class I molecules in the loading complex of ERp57 deficient cells may be due to its effect on class I folding prior to the formation of PLC. They further quantified the total cellular pool of MHC class I molecules and found that the

absence of ERp57 resulted in a decrease of 50-90% in total steady-state class I molecules. However, the reduction in the total cellular pool of MHC class I molecules did not correlate with the cell surface class I expression, indicating ERp57 functions to some extent within the loading complex. Taken together, both CRT and ERp57 seem to play an important role in stabilizing the PLC. It is therefore possible that the elevation of ERp57 is a compensative measure for the defect in CRT deficient cells. Alternatively, unfolded protein response may be activated due to aberrant proteins accumulated in the ER in the absence of CRT(Kaufman, 2002; Harding et al., 2002), which leads to the coordinated synthesis of ER-resident chaperones and enzymes(Mori, 2000), such as BiP(Molinari et al., 2004) and ERp57 (this study). Unfolded protein response could further point to the importance of CRT chaperone function in the maturation of newly synthesized proteins. This result also suggests that CRT and ERp57 may play closely related roles in the quality control of protein maturation, in this case MHC class I assembly. Finally, ERp57 might be dedicated to the reduction of exposed disulfide bonds in the class I molecules due to the incompletely folded or misfolded MHC class I in the absence of CRT. In this way, ERp57 may initiate retrotranslocation and degradation of misfolded class I molecules(Antoniou et al., 2002).

The results in this chapter show that the mechanism by which CRT lowers the peptide concentration threshold did not involve peptide supply, the expression of class I HC and its assembly with β2m, or the chaperone levels in the PLC. The other most likely possibility was impaired peptide loading in CRT deficient cells. To test this possibility, mouse class I K^b binding peptide SIINFEKL was directly delivered into the ER lumen. The cell surface SIINFFEKL:K^b complexes were then assessed by mAb 25-D1.16. Surprisingly, the results showed that ER targeted delivery of SIINFEKL rescued the surface class I expression on CRT deficient cells to the level seen on CRT competent cells (Fig 4.6). Thus, it is reasonable to conclude that CRT deficient cells do not have a global defect in intracellular peptide loading of SIINFEKL peptide after infection by vaccinia virus.

Although peptide loading onto class I molecules within the PLC in the ER contributes to the large majority of class I/peptide complexes at the cell surface, some class I molecules, such as human leukocyte antigen-B (HLA-B) alleles, HLA-B13, B44 and B60, do not seem to use the PLC complex at all for peptide loading(Neisig et al., 1996). Wang et al have suggested that the newly synthesized MHC class I molecules can bind their specific peptide immediately after synthesis without the help of PLC as peptide loading onto class I molecules can occur in the presence or absence of MHC class I-TAP interactions when large amount of class I binding peptides are present in the ER(Paulsson and Wang, 2004). Further evidence for this comes from a study showing only a two fold decrease in surface class I expression even although there is very little recruitment of class I molecules into the PLC in ERp57 deficient cells(Garbi et al., 2006). Furthermore, Williams studied the optimization of HLA-B2705 in tapasin deficient .220 and tapasin-restored .220 cells and found there may be a route of peptide optimization without the need of TAP interaction. Additionally, utilizing the ER competition for TAP association between recombinant rat MHC class I allele RT1-A^a and endogenous class I allele RT1-A^u in the rat C58 thymoma cell line, Powis and colleagues also demonstrated that the loading and optimization of peptide can occur without TAP association(Ford et al., 2004). Taken together, these findings imply that peptides can find their own MHC class I molecules outside the PLC with relatively efficient peptide loading onto MHC class I molecules.

It has been suggested that the dependency of class I molecules on tapasin or TAP/tapasin docking sites for peptide binding is inversely correlated with the size of respective peptide pool capable of binding to class I molecules(Suh et al., 1996; Neisig et al., 1998; Peh et al., 1998). Further evidence exists that the failure of the loading complex to load peptides onto MHC class I molecules can be overcome by increasing peptide concentration in the ER(Lybarger et al., 2001). It is also a formal possibility that an increased concentration of peptide in the ER expressed by vaccinia viruses could compensate for the defect in peptide loading. This was supported by work from several independent studies. For example, in tapasin deficient .220 cells, class I is not associated with TAP. TAP dependent peptide loading is defective in these cells leading to reduced surface class I expression. This defect can be rescued by direct delivery of peptide into

the ER by vaccinia virus. Likewise, a non-conservative point mutation at position 134 of the HLA-A2 HC (A2T134K) renders A2 molecules unable to bind tapasin, TAP and CRT. As a result, A2T134K/β2m complexes are rapidly exported to the cell surface in an unstable, peptide-receptive form. The defect of T134K in endogenous antigen presentation can also be overcome by direct delivery of peptide to the ER using an ER signal sequence(Peace-Brewer et al., 1996; Lewis et al., 1996; Lewis and Elliott, 1998). Although cell surface class I expression on K42 was restored when class I binding peptide was delivered to the ER in a TAP-independent manner, the possibility that the peptide optimisation process is impaired and class I molecules are unable to enrich high affinity peptides for loading in CRT deficient cells still cannot excluded.

Wang et al have proposed the following scenario for MHC class I maturation. Shortly after the newly synthesized HC binds to β2m, a peptide will occupy the peptide binding groove(Neefjes et al., 1993). Peptide loading can occur in the presence or absence of class I-TAP interaction(Paulsson and Wang, 2004). If the peptide is of high affinity, the trimeric complex will be transported to the cell surface(Spiliotis et al., 2000). On the other hand, if the class I receives low-affinity peptide, further action of ER chaperones is required. If the suboptimally loaded class I molecules are able to interact with tapasin, they will not exit the ER as unstable complexes but instead incorporated into PLC to optimize the peptide cargo by exchanging low affinity peptide for high-affinity peptide(Ortmann et al., 1997; Cresswell et al., 1999). Loading of high-affinity peptide dissociates the class I molecules from the PLC and then exits the ER, and traffics to the cell surface(Pentcheva and Edidin, 2001). If the suboptimally loaded class I molecules are unable to interact with tapasin, they will exit the ER followed by either immediate degradation or short-term expression at the cell surface, then followed by endocytosis and degradation(Paulsson et al., 2001).

On the basis of above analysis, I favour the following hypothesis. When high-affinity SIINFEKL peptide was targeted to the ER of CRT deficient cells and over-expressed by the vaccinia virus systems used, the newly formed HC-βm heterodimers will bind the SIINFEKL peptide even in the absence of MHC class I-TAP interaction. TAP association

with class I molecules is not essential for loading peptides as peptides targeted to the ER associate with class I molecules in TAP-deficient cells(Henderson et al., 1992; Wei and Cresswell, 1992; Anderson et al., 1991). However, under normal cellular conditions when peptides are generally present in quantities that are low, loading of MHC class I with stable peptides requires a fully competent loading complex where TAP, tapasin, CRT, ERp57 and class I molecules all interact with one another(Tan et al., 2002). In CRT deficient cells, class I does not interact well with tapasin and TAP in the absence of CRT interactions with class I(Turnquist et al., 2002), leading to lower incorporation of class I molecules into the mature loading complex in CRT deficient K42(Gao et al., 2002). The empty or suboptimally loaded class I molecules that are unable to interact with tapasin are exported in an unstable state and fall apart en route to the cell surface or at the cell surface, resulting in the reduced cell surface levels of MHC class I on CRT deficient cells. On the base of this analysis, CRT would facilitate the class I association with PLC either by recruiting MHC class I into the loading complex or by stabilizing the interaction between class I and tapasin-TAP complex. By doing so, CRT maintains the low peptide concentration threshold required in the ER for efficient antigen presentation.

CRT is important in the assembly of MHC class I molecules by promoting correct folding (Culina et al., 2004) and by inhibiting aggregation of newly synthesised class I molecules(Mancino et al., 2002). CRT possibly also plays a more important role in regulating the release of class I from the PLC and exit to the cell surface, and this mechanism may be important for proper control of peptide loading (Williams et al., 2002a). CRT, like calnexin, only associates with monoglucosylated class I molecules and it has been suggested that this interaction could control the release of class I molecules from the PLC. When class I molecules are properly folded, the terminal glucose is removed by glucosidase-II and this disrupts the interaction between class I and CRT and could lead to the dissociation of CRT-class I-TAP complexes (van Leeuwen and Kearse, 1996a). Glucosidase II and UDP-glucose glycoprotein glucosyltransferase, two ER proteins might distinguish whether MHC class I is loaded with peptide or empty, while CRT modulates the interaction of class I and the PLC (Sadasivan et al., 1996). If class I molecules are not properly folded or not loaded with peptide they will be sensed by UDP-

Glucose glycoprotein glucosyltransferase (GT) and the terminal glucose is then added back onto unloaded class I molecules by GT. In this way, the association of class I molecule with CRT is restored, and unloaded or suboptimally loaded class I returned to the PLC to further optimize its peptide cargo(Wright et al., 2004). This hypothesis is also supported by the observation of fast trafficking and incomplete peptide loading of class I molecules in both CRT deficient cells(Gao et al., 2002) and the T134K mutant in which the association of class I molecules with CRT is disrupted (Lewis et al., 1996; Peace-Brewer et al., 1996). Lewis et al have suggested that the optimization of peptide loading onto class I complexes may be linked to their retention time in the ER. They tested this idea by incubating the cells expressing T134K molecules in the presence of the drug brefeldin A (BFA), which promotes the retention of newly synthesized class I in the ER. They found that the fraction of stable T134K molecules increased dramatically and they proposed that ER retention alone may facilitate the assembly of class I molecules with optimal peptides(Lewis and Elliott, 1998). CRT may therefore aid the retention of the empty or suboptimally loaded class I complexes in the ER for peptide optimization(Elliott and Williams, 2005). In this way CRT lowers the ER peptide concentration needed for antigen presentation.

One further property of CRT is its ability to bind to peptides transported into the ER by TAP (Spee and Neefjes, 1997). This binding can be enhanced under the condition of cell stress (Rizvi et al., 2004). Peptides of free form probably do not exist in the ER as they are degraded very quickly by ER peptidases unless they are protected, most likely by their association with chaperones, such as CRT(Paz et al., 1999; Spee and Neefjes, 1997; Basu and Srivastava, 1999; Reits et al., 2003). As a component of the PLC, CRT might be able to bring peptides into the proximity of class I molecules and facilitate their loading. CRT chaperoned peptides would also remain in the ER longer to increase the chances of loading onto MHC class I molecules. In this way CRT may help to increase the efficiency of peptide loading and lower the concentration of peptides required for proper antigen presentation.

CRT is an ER resident protein, but has been observed elsewhere, such as on the cell surface (Arosa et al., 1999; Wiest et al., 1997). It can not be excluded therefore that CRT, like some other members of heat shock protein family, could bind to peptides in the cytosol and enrich them for further processing. It would be interesting to deliver extended peptides containing a specific epitope to the cytosol to investigate whether there is a difference in epitope presentation between CRT deficient cells and wild type cells.

Facilitating peptide loading onto the class I complex and chaperoning peptide might also be a mechanism for the adjuvant property of CRT. A study done by Binder and colleagues showed that CRT associated with peptide in the ER, acted as a nonspecific adjuvant. Peptides chaperoned by CRT or other Hsps were a necessary and sufficient source of antigen for transfer to APCs for priming CTL response(Binder and Srivastava, 2005). Although a direct need for chaperones in peptide delivery to MHC class I molecules in the ER has not been uncovered, various peptide-chaperone complexes have been tested in tumor vaccines with some success. They are apparently able to deliver peptides to class I molecules through a process called cross-presentation(Srivastava, 2002; Singh-Jasuja et al., 2000; Tamura et al., 1997). In vitro incubation of such CRT-antigenic peptide complexes with APCs allows cross-presentation of the peptide to CTLs. The immunogenicity of the CRT-peptide complexes is attributed to the peptide chaperoned by the CRT molecule(Basu and Srivastava, 1999; Nair et al., 1999).

In the absence of CRT, there were still a substantial amount of class I molecules recruited into the PLC. CRT deficient cells were able to present cytosolically-derived peptides at the cell surface for recognition by T cells, albeit with reduced efficiency. As discussed earlier, however, the interaction of CRT with HC has been demonstrated to be a prerequisite for the association of class I with TAP via tapasin. For example, the drug castanospermine, which prevents the generation of the specific ligand recognized by CRT and CNX, has been shown to inhibit the interaction between MHC class I molecules and TAP(Sadasivan et al., 1996). Additionally, Harris et al showed that the mutation of the amino acid at position 86 alters the Asn-linked glycosylation site, and leads to PLC

disruption through poor CRT association(Harris et al., 1998; Harris et al., 2001). This, therefore, leads us to consider that there may exist a redundent chaperone with roles like CRT in mediating MHC class I mediated antigen presentation in CRT deficient K42 cells.

In general, the ER contains a powerful and redundant system with chaperones able to substitute for each other and cooperate in different ways to assist protein folding and assembly. For example, in a cell line that lacked calnexin, the class I HC was able to assemble with β2m, associate with TAP/tapasin complex, receive peptide, and exit the ER normally(Sadasivan et al., 1995; Scott and Dawson, 1995; Prasad et al., 1998). Accordingly, it has been proposed that there is redundancy in the ER and that other chaperones can perform the same function as CNX in its absence. Immunoglobulin binding protein (BiP) has been suggested to be a possible candidate as BiP has been shown to associate with free human class I HC alleles in $\beta 2m^{-/-}$ cells and its expression is upregulated in calnexin deficient cells(Paulsson et al., 2001). CRT, which has homology in some domains to calnexin and shares lectin-like activity with it(Michalak et al., 1992; Peterson et al., 1995), is another candidate for adopting some of the functions performed calnexin. Chaperone redundancy may account for the lack of any observed effect of calnexin absence on class I assembly, transport and surface expression in calnexin deficient cells. The "backup" mechanisms existing within the ER quality control system assures the correct folding and assembly of MHC class I molecules in cells without calnexin.

Due to the similarity in glycan binding specificity and overall structure, CRT and calnexin have been proposed to have redundant activities. When one was absent, binding of substrate glycoproteins to the other increased. A dramatic loss of stringency in the ER quality control is only observed when substrate access to both CRT and calnexin is prevented(Molinari et al., 2004). The most likely redundant protein carrying out the chaperone function of CRT in CRT deficient cells is therefore calnexin. Calnexin has been shown to interact with the glycan moities on the class I HC and assists the assembly

of class I molecules(David et al., 1993; Degen and Williams, 1991; Degen et al., 1992; Hochstenbach et al., 1992). In addition, CNX has been detected in complexes with mouse class I HC-β2m dimers(Degen et al., 1992; Jackson et al., 1994). Furthermore, calnexin has been found to be part of PLC in K42 cells(Gao et al., 2002). Accordingly, it has been suggested that calnexin and CRT have redundent functions in the assembly and expression of MHC class I molecules(Danilczyk et al., 2000; Sadasivan et al., 1995; Solheim et al., 1997). Calnexin, like its luminal homologue CRT, possesses a proline rich central P domain, which has been shown to bind to ERp57(Leach et al., 2002) and has been identified to be the lectin site of CRT capable of binding oligosaccharide(Vassilakos et al., 1998). One possible model to account for the phenotype of CRT deficient K42 cells is that calnexin, interacting with both class I molecules and ERp57, may act like CRT to assist the formation of PLC and modulate the peptide loading. This could explain why MHC class I molecules are still incorperated into the PLC of CRT deficient cells, loaded with cytosolically-derived peptides, and presented them at the cell surface for recognition by T cells(Gao et al., 2002). Nonetheless, it is possible that calnexin is not as competent as CRT in assisting the formation of PLC, providing quality control of class I folding and recycling, and facilitating peptide loading onto class I through its capacity of peptide binding. Accordingly, CRT deficient K42 cells have impaired assembly, suboptimal peptide loading, and reduced cell surface expression of class I molecules.

In summary, CRT is an important molecule for maintaining a lower threshold of peptides in ER and efficient antigen presentation. Possible mechanisms by which CRT achieves this role would be to assist the formation of PLC; to provide quality control of class I folding and recycling; and/or to facilitate peptide loading onto class I through its capacity of peptide binding, or a combination of these three activities.

Chapter 5 Investigating the domain functions of CRT in MHC class I antigen presentation

5.1 Introduction

Peptide loading onto class I HC-β2microglobulin heterodimers is critical for both the structural stability and the function of MHC class I molecules and is assisted by the PLC(Cresswell et al., 1999). Structurally, CRT is an important member of the PLC and seems to play a central role in the formation of the PLC by interacting with other component of the complex. CRT associates with MHC class I after HC is assembled with β2m, and the association of CRT with MHC I is not dependent on either TAP or tapasin, but appears to be a prerequisite for the association of class I with TAP via tapasin(Sadasivan et al., 1996; Solheim et al., 1997; Harris et al., 1998; Turnquist et al., 2002). Disrupting the CRT interaction with class I HC by either mutation of the residue at position 86 to alter the Asn-linked glycosylation site(Harris et al., 1998; Harris et al., 2001; Turnquist et al., 2002), or using the drug castanospermine to block the action of glucosidase I and II to prevent the generation of the monoglucosyl-glycan recognized by CRT has been shown to cause PLC disruption through poor association with class I(Sadasivan et al., 1996; Elbein, 1991; Harris et al., 1998; Harris et al., 2001). Furthermore, Gao et al. have found that less class I molecules were incorporated into the PLC in the absence of CRT(Gao et al., 2002). In addition to its association with class I HC, CRT has been found to interact with ERp57 through the tip of its P-domain(Frickel et al., 2002; Leach et al., 2002). It was suggested that CRT recruits ERp57 providing an "attachment" site for this enzyme to chaperone (disulfide bond formation) newly synthesised glycoproteins. ERp57 chaperone activity is greatly increased when complexed with CRT(Zapun et al., 1998).

Functionally, CRT has been suggested to stabilize and retain the HC-β2m heterodimer in the ER until the antigenic peptide is loaded(Harris et al., 1998). Studies have shown that CRT preferentially binds to open conformations of MHC class I and does not associate with the class I after peptide-induced folding(Harris et al., 1998; Sadasivan et al., 1996).

Furthermore, CRT deficient cells have reduced cell surface class I expression and fail to present the majority of epitopes tested to specific T cells, which suggests that CRT is critical for MHC class I assembly and its subsequent antigen presentation(Gao et al., 2002). In chapter 4, it was shown that higher concentration of antigen in the cytosol is required for specific T cell stimulation in CRT deficient cells than in CRT competent cells, whereas TAP function and protein expression level was the same. It was proposed that CRT functions to maintain the low peptide concentration threshold required in the ER for efficient antigen presentation.

To learn more about how CRT contributes to MHC class I antigen presentation and how the function of CRT relates to its structure, full length CRT or one of three structurally and functionally distinct domains, N-domain, the central P-domain, and the C-domain, were introduced into both CRT deficient cells (K42) and CRT competent cells (K41) to examine the role of CRT and its truncated forms in the formation of the PLC and MHC class I antigen presentation. An assessment was also made on the effect of such expression on cellular functions, such as peptide loading, TAP function, and expression of other members of PLC, and the possible underlying mechanisms.

5.2 Results

5.2.1 Generation of stable cell lines expressing CRT and its fragments

5.2.1.1 Constructs design

To further dissect the role of calreticulin in MHC class I antigen processing, different fragments of human calreticulin with functional domains (Fig. 5.1a) were cloned into a mammalian cell expression vector along with a full length of CRT as control. Human CRT was chosen because CRT is a highly conserved protein, with the nucleotide sequences of the human and mouse genes showing greater than 70% identity, and 95% amino acid identity existing between human and mouse forms of protein with only one amino acid difference in P-domain between human and mouse type CRT (Appendix). In order to maintain CRT fragments in the ER, the genes were cloned into a vector with built-in ER leader and retention signals. For the convenience of detection, a myc sequence was attached to the C-terminal of each fragments before the stop codon. A two-step strategy of cloning was adopted: first, PCR products of each fragment were cloned into a TOPO vector and then they were subcloned into the vector containing both ER leader sequence and retrieval signal. The genes encoding full length human CRT and its P, PC and NP domains were amplified by PCR using appropriate primers (listed in materials and methods) with 5' flanking Pst I and 3' flanking Not I restriction sites (Fig. 5.1 b). Then the PCR products were separated in an agarose gel and the PCR band was gel-extracted and cloned into the polylinker of the pcDNA3.1 TOPO TA vector (Fig. 5.2). Next, the Pst I/Not I cDNA fragment, containing cDNA encoding full-length human CRT or different fragments of calreticulin, were excised from TOPO TA vector and inserted into the Pst I/Not I restriction sites of pEF/myc/ER vector to generate pEF/myc/ER-P, pEF/myc/ER-PC, pEF/myc/ER-NP and pEF/myc/ER-CRT (Fig. 5.3). The sequences of all constructs were confirmed by sequencing.

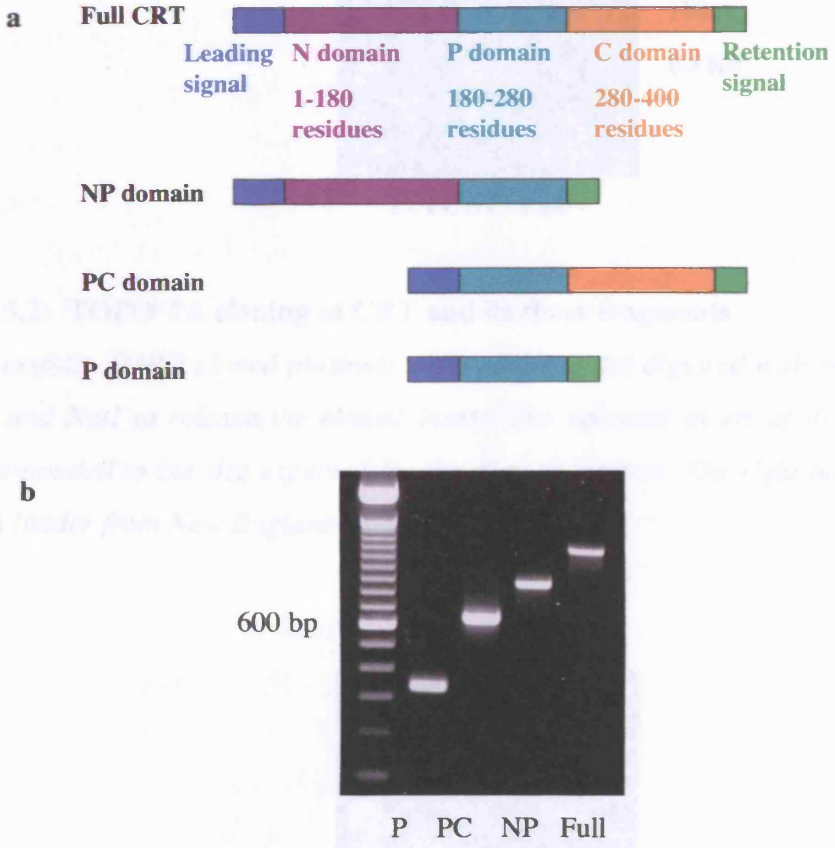


Fig. 5.1: PCR CRT and its P, PC, NP fragments

a: Schematic representation of full length calreticulin and its NP, PC, P fragments used in this study.

b: Genes were successfully amplified from plasmids containing calreticulin cDNA as outlined in Table 4.2 by PCR. PCR products were run on 1% Agarose gels and stained with ethidium bromide (EtBr) and photographed on a UV transilluminator. P is the P-domain fragment and its size is 330 bp. PC is the fragment with P and C domains of calreticulin and the size is 645 bp. Similarly, NP represents N and P domains of calreticulin and the size is 922 bp. The size of full CRT gene is 1237 bp. The DNA ladder is a 100 bp ladder (Invitrogen).

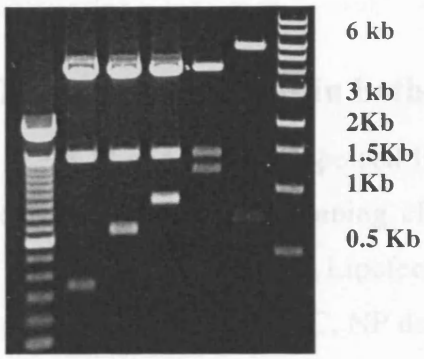


Fig. 5.2: TOPO TA cloning of CRT and its three fragments

Successfully TOPO cloned plasmids were purified and digested with restriction enzymes PstI and NotI to release the cloned insert. The released insert of the individual clone corresponded to the size expected for the gene of interest. The right hand lane is a 1 Kb DNA ladder from New England, Biolab.

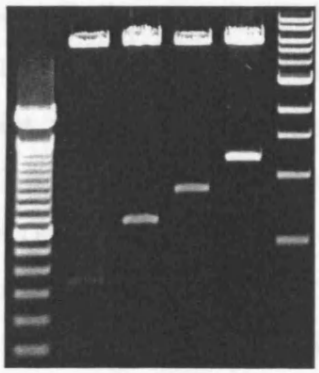


Fig. 5.3: Cloning of CRT and its three fragments into pEF/myc/ER

The pEF/myc/ER-P, pEF/myc/ER-PC, pEF/myc/ER-NP, pEF/myc/ER-CRT vectors were digested with restriction enzymes PstI/NotI. The expected sizes have been observed for all cloned genes. The right lane is a 1 Kb DNA ladder from New England, Biolab.

5.2.1.2 Expression of CRT and its fragments in both K41 and K42 cells

The Calreticulin-deficient cell line-K42 and its wild type cell line-K41 were transfected with individual pEF/myc/ER expression vectors containing cDNAs encoding the full-length human CRT or its P, PC and NP domains using a Lipofectamine based transfection kit (Invitrogen). To verify that the full-length and P, PC, NP domains of CRT expressed with this vector would be directed to the ER, K42 and K41 cells were transfected with GFP expression control vector pEF/myc/ER-GFP and the expression pattern of GFP was analysed by immunofluorescence confocal microscopy. An ER-distribution fluorescence pattern was observed in these cells (Fig. 5.4), suggesting the recombinant CRT and its domains would be targeted correctly to the ER of K42 and K41 cells.

To establish stable lines for individual transfectants, G418 was added to the medium of cells transfected with vector containing the G418 resistant gene. Since both cell lines came from a G418 resistant background, a very high concentration of drug was necessary for selecting stable transfected cell line. 6mg/ml G418 was used for selection. The expression of myc tagged CRT and its domains in transfected K42 and K41 cells were first confirmed by western blot using anti-myc antibody against the c-myc epitope. Positive clones expressing CRT and its P, PC and NP domains were obtained with both K42 (Fig. 5.5a) and K41 cells (Fig. 5.5b). Results showed that the anti-myc antibody reacted with a 60 kDa protein in lysates of full-length CRT transfected k42 and K41 cells confirming the expression of full-length CRT. The anti-myc antibody reacted with a 30 kDa protein in lysates of P domain transfectants and with a 45kDa protein in lysates of PC, NP transfectants demonstrating the expression of myc tagged P, PC and NP domains of CRT in these cells.

The cell lysates were further analysed by Western blot using an anti-CRT monoclonal antibody specific for human CRT. This antibody reacted with a 60 kDa protein in full-length CRT transfected cells and a 45 kDa protein in NP domain transfected cells (Fig.

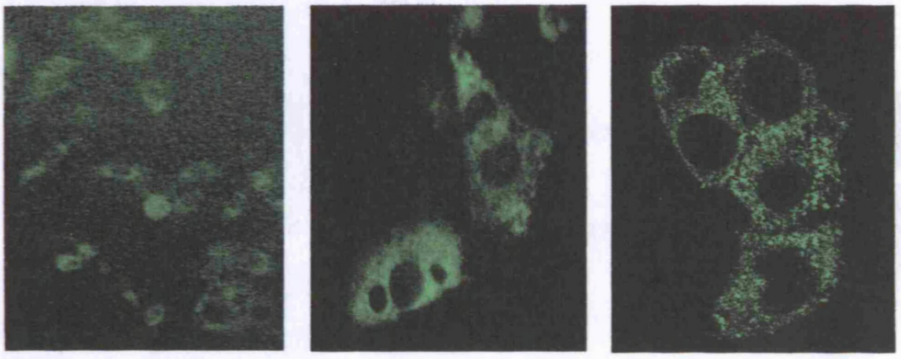
5.5c) but not P and PC encoding products. This suggests that the monoclonal antibody is N-domain specific.

For simplicity, stably transfected K42 and K41 cell lines expressing full length human CRT and its P, PC, NP domains will be referred to as K42-CRT, K42-P, K42-PC and K42-NP, and K41-CRT, K41-P, K41-PC and K41-NP respectively. To make sure that all stable cell lines were genuinely calreticulin positive (K41) or negative (K42), western blots for mouse native CRT was also performed on these eight positive colones. K41 cells and the four K41 clones expressing recombinant CRT and its domains were confirmed as CRT positive, while K42 cells and K42 clones expressing recombinant CRT and its domains were confirmed as CRT deficient in Fig. 5.5 d and e.

Phase contrast analysis of K41, K42 and P, PC and NP domain of CRT transfected K42 cells revealed that the morphological appearance of the wild-type (K41) and calreticulin-deficient (K42) cells is indistinguishable and typical of fibroblasts. The cell lines all attached firmly to plastic although K42 cells grew a bit slower than K41 cells.

The morphological appearance of P, PC and NP domain transfected K42 cells is different from that of the wild-type (K41) and calreticulin-deficient (K42) cells. The K42 cells expressing CRT fragments but not full length CRT were more rounder than K41, K42 cells (Fig. 5.6). The reasons for morphological difference require further investigation.

K42



K41

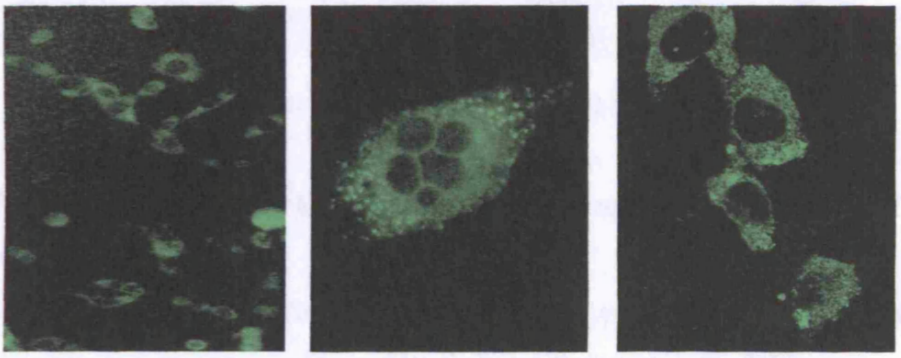


Fig. 5.4 ER distribution of GFP expressed using pEF/myc/ER/GFP vector

K42 (upper panel) and K41 cells (lower panel) were transfected with expression vectors encoding GFP with the same vector (pEF/myc/ER) used for the expression of P, PC, NP, and full length CRT.

Left panel: low magnification of fluorescence microscopy (10X)

Middle panel: high magnification of fluorescence microscopy (100X)

Right panel: confocal microscopy

Intracellular location of recombinant CRT analysed by immunofluorescence microscopy revealed a reticular, perinuclear pattern characteristic of ER.

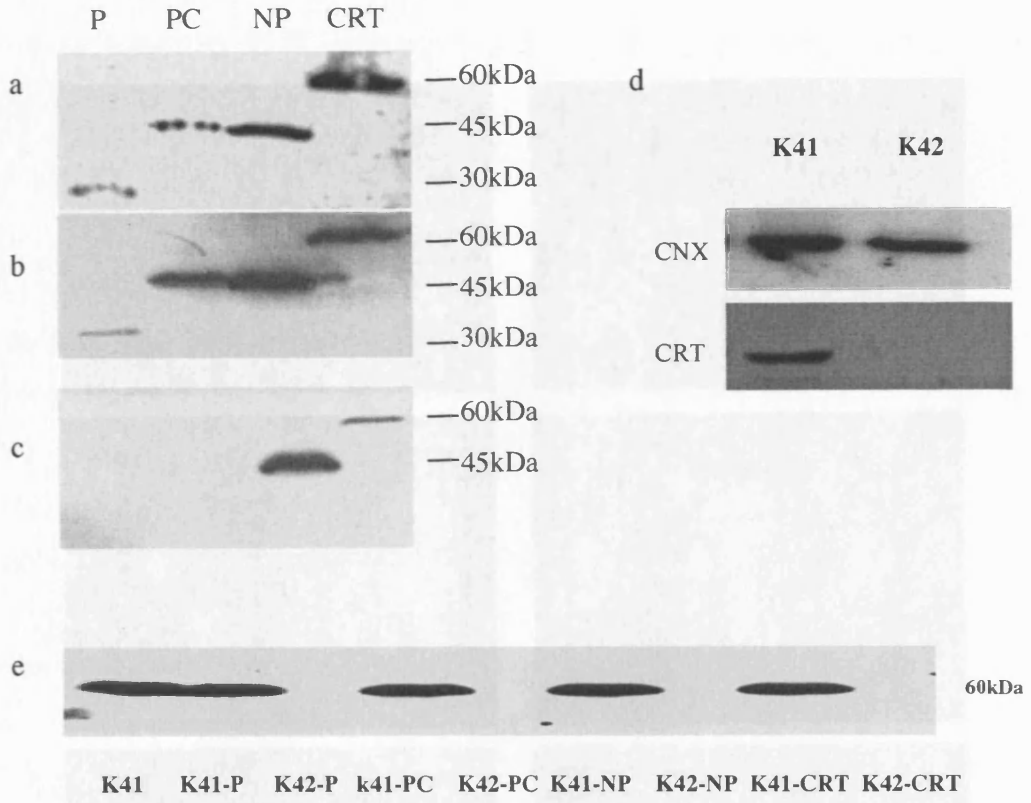


Fig. 5.5 Establishment of cell lines expressing full-length recombinant CRT and its fragments

The calreticulin deficient cell line K42 and its wild type counterpart K41 cells were transfected with human calreticulin and its P, PC, NP domains cloned into a vector with both ER leader sequence and retention signal. Cells were lysed in 1% Triton X-100, and the lysates were separated by 12.5% SDS-PAGE, transferred to nitrocellulose membrane, and probed with anti-myc monoclonal antibody (a and b), anti-calreticulin monoclonal antibody from Stressgen (c), and a rabbit anti-calreticulin antibody (d and e), which recognizes only native CRT, not recomninant CRT.

Western blot with anti-myc mAb confirms transfection of P, PC, NP and full length CRT into K42 (a) and K41 (b) cells. Anticalreticulin antibody (Stressgen) can only recognize the NP and full length CRT, not the P and PC fragments (c)

K41 cells and its clones expressed mouse native calreticulin. There was no expression of native calreticulin in K42 cells and its clones as expected (d and e).

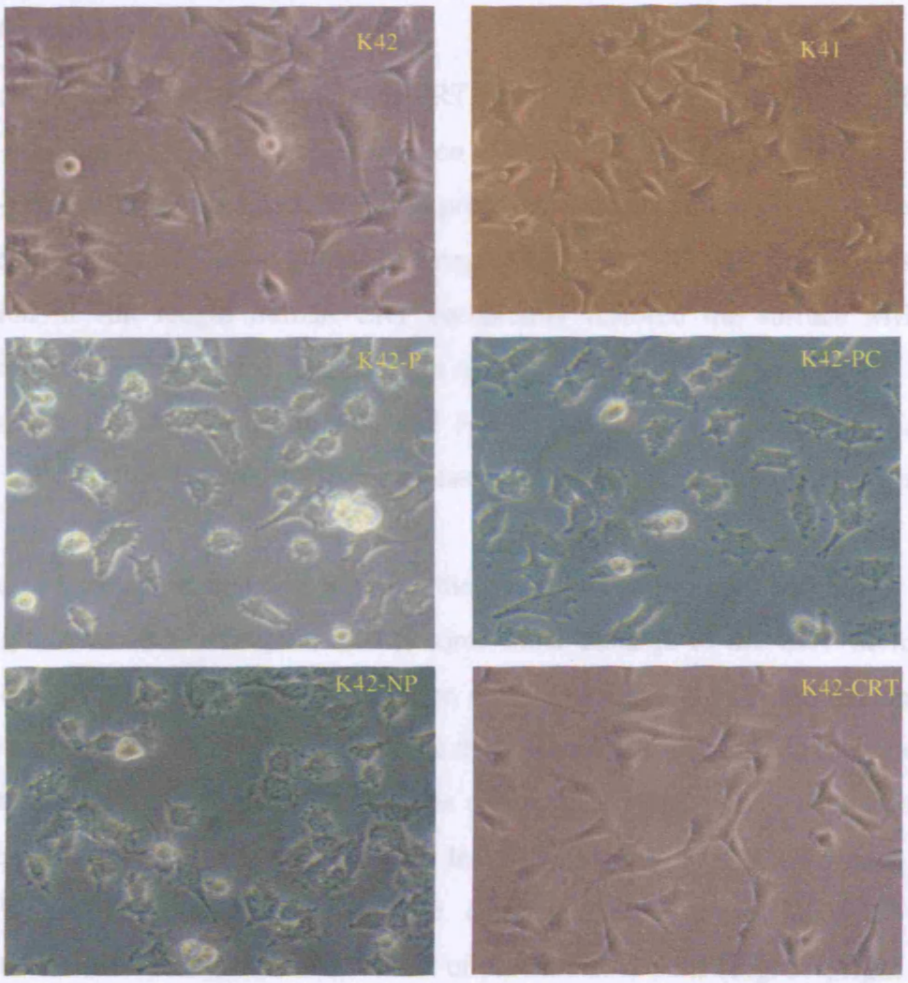


Fig. 5.6 Phase contrast morphological observation of K42 and its transfectants

The morphological appearance of the wild-type (K41) and calreticulin-deficient (K42) cells is indistinguishable and typical of fibroblasts. The cell lines all attached firmly to plastic.

The morphological appearance of of P, PC and NP domain of CRT transfected K42 cells is different from that of the wild-type (K41) and calreticulin-deficient (K42) cells. The cells of CRT fragments K42 transfectants are more round than K41 and K42 cells.

5.2.2 Cell-surface class I expression is reduced by truncated CRT, while full-length CRT corrects the class I expression defect in K42 (CRT-/-) cells

To determine the effect of recombinant CRT and its P, PC, NP fragments on MHC class I antigen presentation, cell-surface expression of mouse class I (H-2K^b) on transfected K42 (CRT-/-) cells was investigated. H-2K^b expression was detected with Y3, a conformation dependent monoclonal antibody (mAb) recognizing H-2K^b. The results demonstrated that expression of full length human CRT completely restored the surface MHC class I expression in CRT deficient K42 cells to the level seen in CRT competent K41 cells, whereas, the introduction of P, PC, and NP fragments of CRT into the ER of cells without CRT further reduced surface expression of MHC class I (Fig. 5.7, left panel).

These results raised the question whether the individual fragments had a major impact on class I synthesis and folding or caused some other damage to the CRT deficient cells, which lead to down-regulation of surface class I expression. If this is the case, the truncated CRT would be expected to cause the same reduction of MHC class I expression in calreticulin positive cells. Therefore, the cell surface MHC class I expression of CRT competent K41 cells transfected with full length and truncated CRT was also examined by mAb Y3. Surprisingly, the surface class I expression in all K41 (CRT+/+) transfectants was found at the same as that of parental K41 cells (Fig. 5.7, right panel).

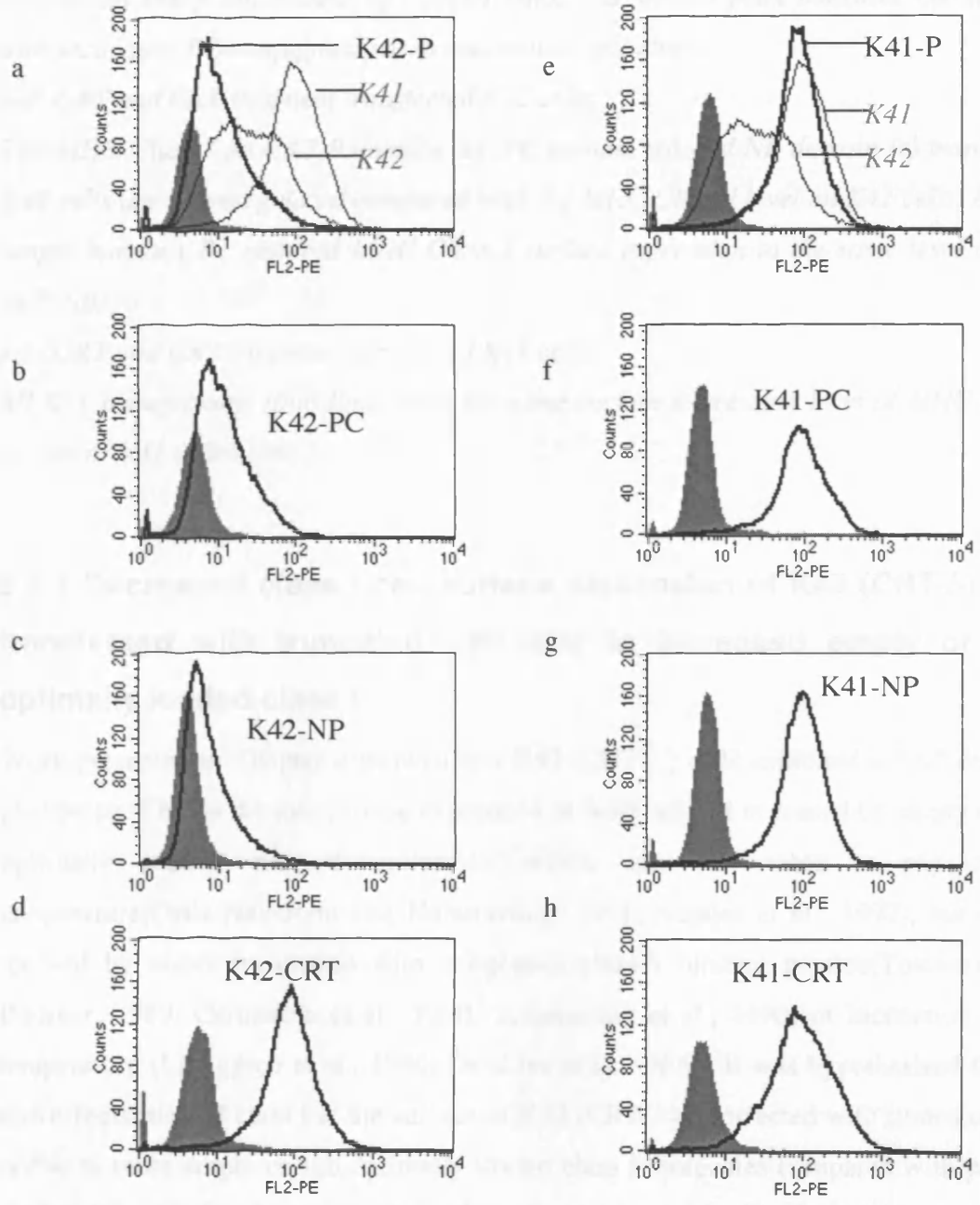


Fig. 5.7 Down regulation of MHC class I moleucules by truncated CRT in the absence of calreticulin

K42 and K41 cells were transefcted with full length CRT and its P, PC, NP fragments and assigned as K42-P, K42-PC, K42-NP, K42-CRT and K41-P, K41-PC, K41-NP, K41-CRT. Cell-surface expression of K^b was detected with mAb anti H-2K^b(Y3) and PE

conjugated sheep anti-mouse IgG (solid line). The shaded peak indicates the staining with secondary PE-conjugated sheep anti-mouse IgG alone.
a-d: CRT and CRT fragment transfected K42 cells.
The MHC Class I on CRT P domain (a), PC domain (b) and NP domain (c) transfected K42 cells are downregulated compared with the MHC Class I level on K42 cells. But full length human CRT restored MHC Class I surface expression to the same level as K41 cells (d).
e-h: CRT and CRT fragment transfected K41 cells.
All K41 transfectants (thin line) show the same surface expression level of MHC class I as that of K41 (thick line).

5.2.3 Decreased class I cell surface expression of K42 (CRT-/-) cells transfected with truncated CRT due to increased empty or sub-optimally loaded class I

Work presented in Chapter 4 showed that K42 (CRT-/-) cells exhibited a TAP deficient phenotype. That is the low surface expression of MHC class I is caused by empty or sub-optimally loaded class I molecules, which are not stable at physiological temperature(Ortiz-Navarrete and Hammerling, 1991; Neefjes et al., 1992), but can be rescued by either incubation with exogenous class I binding peptide(Townsend and Bodmer, 1989; Cerundolo et al., 1990; Schumacher et al., 1990) or incubation at low temperature (Ljunggren et al., 1990; De Silva et al., 1999). It was hypothesised that the down-regulation of class I at the surface of K42 (CRT-/-) transfected with truncated CRT is due to more empty or sub-optimally loaded class I molecules compared with parental K42 (CRT-/-) cells. To test this idea the cells were pulsed with chemically synthesised mouse class I H-2K^b-high-affinity binding peptide- SIINFEKL and the extent to which cell surface H-2K^b molecules could be occupied by this immunodominant - H-2K^b binding peptide was analysed by staining for cell surface H-2K^b with a conformation-dependent class I antibody Y3. The results in Fig. 5.8 showed that the expression level of surface MHC class I on CRT fragment K42 transfectants all increased. However, there was only a slight increase in surface expression of class I on K42-CRT cells indicating

that most of the surface class I molecules on this cell line were already loaded with high-affinity peptides. Thus, the H-2K^b molecules on the surface of truncated CRT transfected K42 were much more receptive to exogenous peptide than those on the surface of full length CRT-reconstituted K42. By contrast, there was no change of MHC class I expression level on the cells pulsed with irrelevant control peptide GILGFVFTL, which confirmed that the restoration of surface class I was peptide specific. The marked receptivity of H-2K^b molecules expressed on truncated CRT transfected K42 cells suggested that these molecules were empty or complexed with suboptimal peptides easily exchanged for the high-affinity SIINFEKL determinant(Chen et al., 1994). These results suggest that most MHC class I molecules in K42-P, K42-PC and K42-NP cells are empty sub-optimally loaded class I molecules, which would fall apart either at the cell surface or en route to the cell surface unless they encounter exogenous peptides to which they can bind.

A prediction from the above conclusion is that suboptimal peptide ligands would dissociate rapidly at 37 °C, but can be stabilized at 26°C. To this end, the cells were incubated overnight at either 26 °C or 37 °C and the surface class I expression was assayed by flow cytometry analysis with mAb Y3. As shown in Fig. 5.9, the surface class I expression of K42-P, K42-PC and K42-NP cells cultured at 26 °C dramatically increased compared with that of cells incubated at 37 °C. In contrast, the same class I expression level can be seen on K42-CRT cells cultured at either 26 °C or 37 °C. Here TAP deficient RMA-S and TAP competent RMA cells were used as an experiment control as it has been previously demonstrated that the majority of class I molecules at the surface of RMA-S cells were empty due to peptides shortage in the ER, and these empty class I could be stabilized and expressed on the cell surface at 26 °C(Ljunggren et al., 1990; De Silva et al., 1999). Based on these data, it can be concluded that the reduction in the surface class I expression of K42-P, K42-PC and K42-NP was caused by high levels of empty or sub-optimally loaded class I molecules.

In order to further show whether the MHC class I molecules expressed on K42-P cultured at 26°C were truly empty or sub-optimally loaded class I molecules, two flasks of K42-P

were cultured at 26°C and one flask at 37°C for 24 h. Then one flask of K42-P cultured at 26°C were transferred to 37°C incubator and cultured for further 5 min. Then all the cells were stained by mAb Y3 and analyzed by flow cytometry. Fig. 5.10 showed that the amount of class I molecules expressed by K42-P cells cultured first at 26°C then 37°C was lower than that of cells incubated at 26°C, but still higher than that of cells cultured at 37°C, which further indicated that the MHC class I molecules expressed on the surface of K42-P cultured at 26°C were indeed suboptimally loaded MHC class I molecules, which dissociated rapidly at the cell surface when temperature was returned to 37 °C.

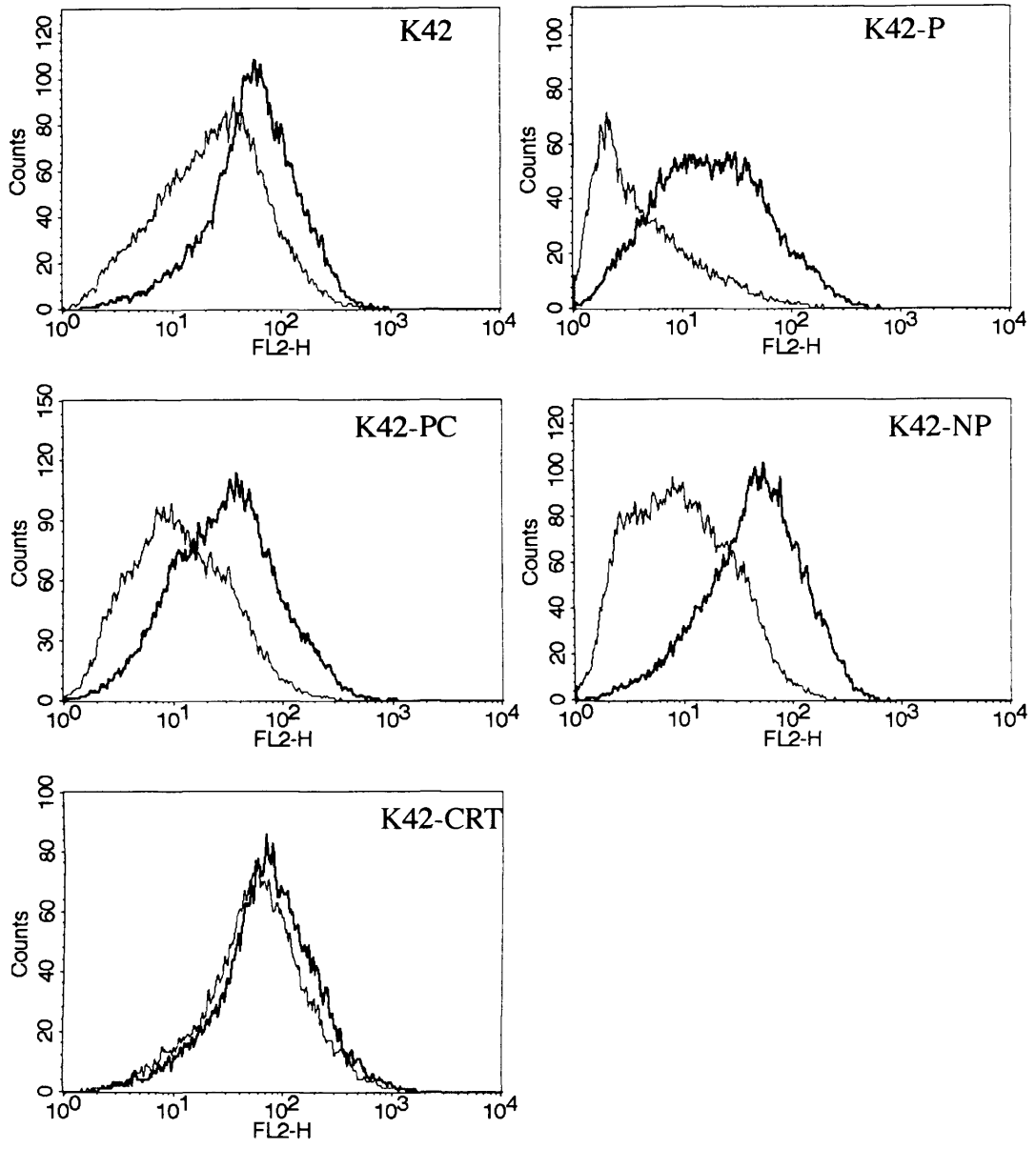


Fig. 5.8 Incubation with exogenous peptide increased surface class I expression

Cells were pulsed with H-2K^b binding peptide SIINFEKL (thick line), with flu peptide GILGFVFTL (thin line) as control. After washing away free peptides, cells were stained by Y3 antibody and analysed by flow cytometry.

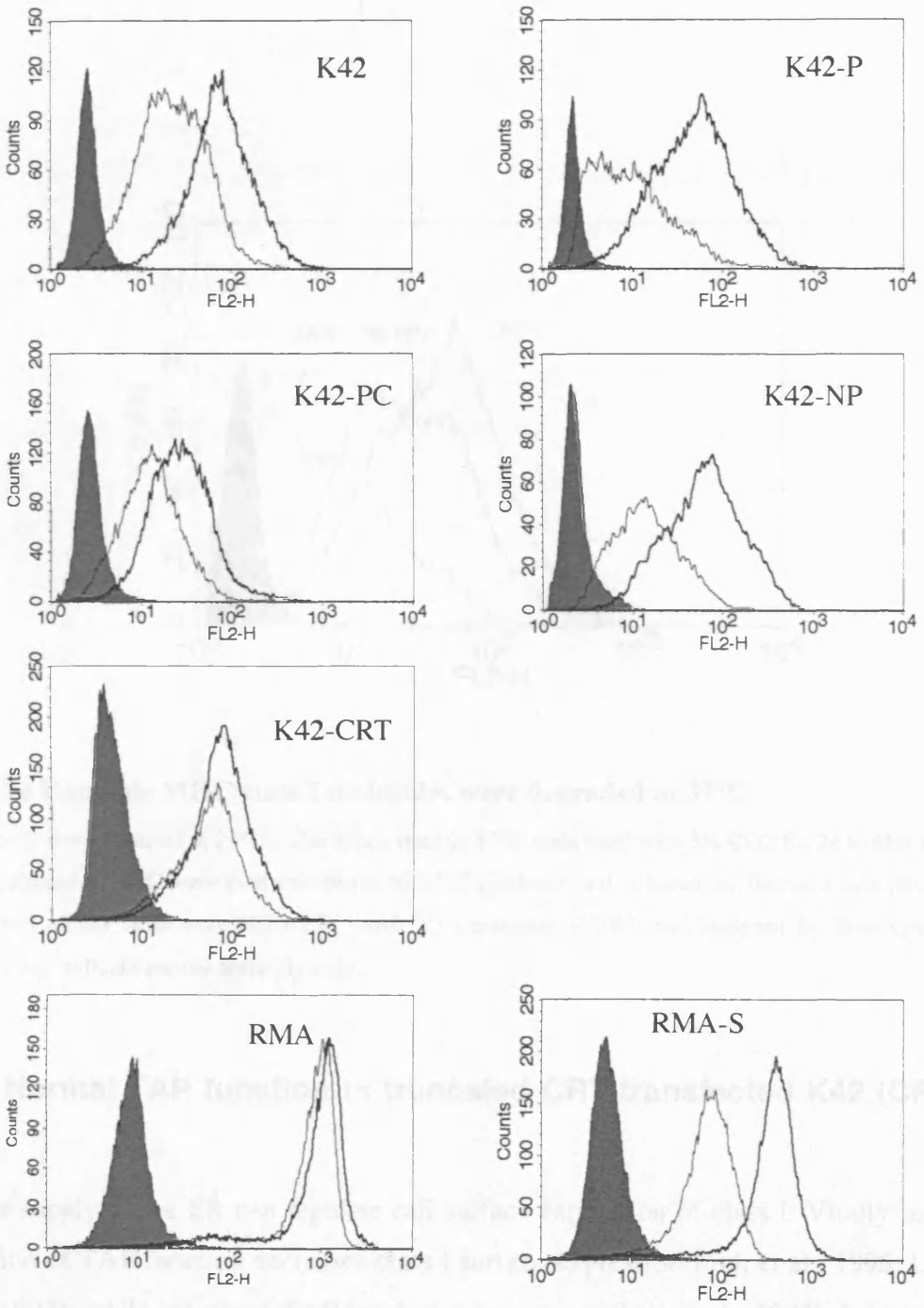


Fig. 5.9 Empty class I comes out in the cold

Cells cultured at 26°C (thick line) and 37°C (thin line) were evaluated by flow cytometry using mAb Y3. Shaded areas indicate second antibody only. Results shown are representative of three independent experiments.

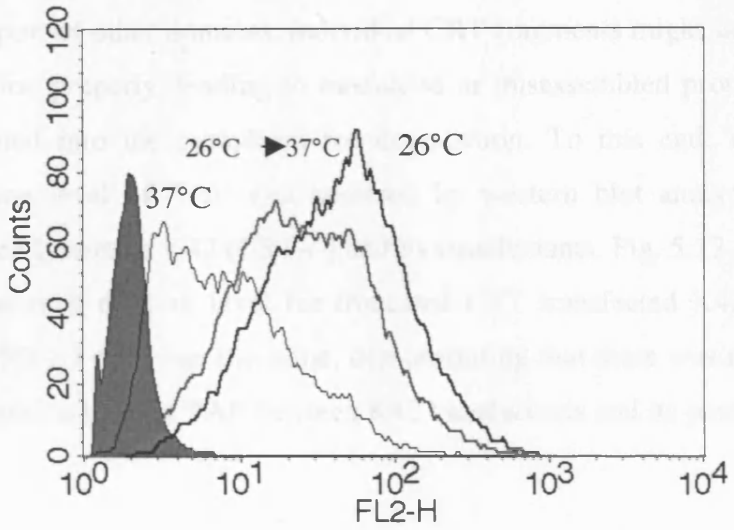


Fig. 5.10 Unstable MHC class I molecules were degraded at 37°C

K42-P cells were cultured at 26°C (thick black line) or 37°C (thin line) with 5% CO2 for 24 h. One flask of K42-P cultured at 26°C were then transferred to 37°C incubator and cultured for further 5 min (thick grey line). Then all the cells were stained by mAb Y3 (recognize H-2K^b) and analyzed by flow cytometry. Shaded areas indicate second antibody only.

5.2.4 Normal TAP function in truncated CRT transfected K42 (CRT-/-) cells

Peptide supply to the ER can regulate cell surface expression of class I. Virally induced inhibition of TAP function decreases class I surface expression(Fruh et al., 1995; Lehner et al., 1997), while increased TAP levels can increase it(Russ et al., 1995). It is possible that the increased empty or sub-optimally loaded class I molecules in K42-P, K42-PC and K42-NP cells might be due to peptide supply in the ER caused by dysfunction of TAP either at the protein level or at the functional level. CRT is an important molecular chaperone and plays a critical role in the folding, oligomeric assembly and quality control of newly synthesized glycoproteins(Peterson et al., 1995; Wada et al., 1995). Moreover,

although the N domain and P domain of CRT are responsible for its chaperone function(Nakamura et al., 2001b), studies suggest that the chaperone function of N and P domains are modulated by Ca^{2+} binding to the C-domain of CRT(Corbett et al., 1999; Corbett et al., 2000; Lodish and Kong, 1990; Lodish et al., 1992; Booth and Koch, 1989). Without the support of other domains, individual CRT fragments might not perform their chaperone function properly, leading to misfolded or misassembled proteins, which are eventually targeted into the cytoplasm for degradation. To this end, the steady-state protein expression level of TAP was assessed by western blot analysis using serial dilution of the cell lysates of K42 (CRT-/-) and its transfectants. Fig. 5.12 showed that the band intensity at each dilution level for truncated CRT transfected K42 (CRT-/-) and parental K42 (CRT-/-) cells was the same, demonstrating that there was no difference in the protein expression level of TAP between K42 transfectants and its parental cells.

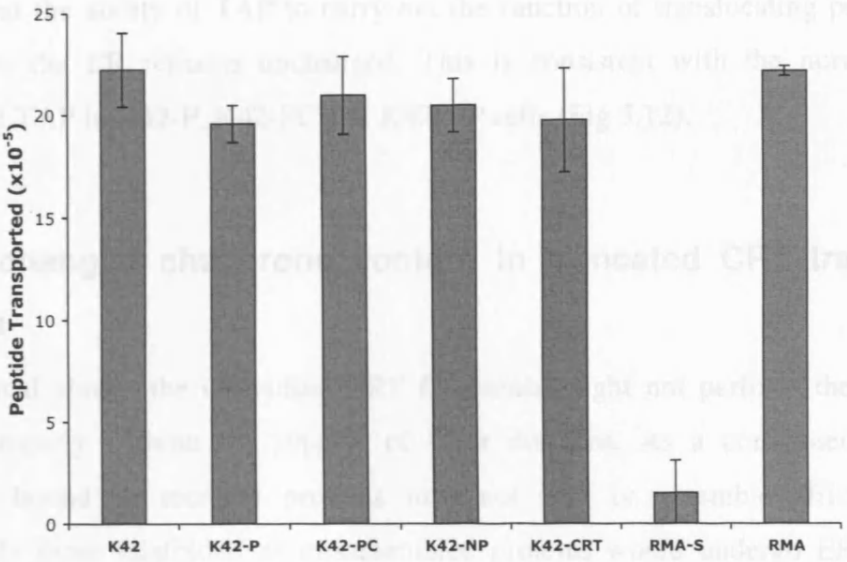


Fig. 5.11 TAP function is normal in truncated CRT transfected K42 cells

Cells were permeabilized and incubated with FITC-labelled peptide (RRYQNSTEL), which were subsequently transported by TAP from cytosol into the lumen of ER, where the peptides were glycosylated. Cells were then lysed, and glycosylated peptides bound to Con-A beads were eluted. The fluorescent intensity of FITC labelled RRYQNSTEL was quantified using a fluorescence spectrometer as described in material and methods. Data are presented as the percentage of peptides transported and shown as means ±SD of three independent experiments.

Next, a peptide transport assay was carried out to compare TAP function in K42-P, K42-PC and K42-NP with that of parental K42 (CRT-/-) cells. Cells were permeabilized with streptolysin O, then incubated with FITC labelled peptide RRYQNSTEL containing an N-linked glycosylation motif. In the presence of functional TAP, the peptide was translocated into the ER, where it is glycosylated and can be recovered by binding to concanavalin A-Sepharose beads(Neefjes et al., 1993). The fluorescent intensity of eluted peptide from concanavalin A beads were quantitatively analysed by fluorescence spectrometer. As shown in Fig. 5.11, an equal amount of FITC labelled peptides were

transported into the ER for all the truncated CRT K42 transfectants and K42 cells. This suggests that the ability of TAP to carry out the function of translocating peptide from cytosol into the ER remains unchanged. This is consistent with the normal protein synthesis of TAP in K42-P, K42-PC and K42-NP cells (Fig 5.12).

5.2.5 Unchanged chaperone content in truncated CRT transfected K42 cells

As mentioned above, the individual CRT fragments might not perform the chaperone function properly without the support of other domains. As a consequence of this, membrane bound or secreted proteins may not fold or assemble efficiently, and subsequently these misfolded or misassembled proteins would undergo ER-associated protein degradation. The other components of the MHC class I loading complex, such as calnexin, tapasin, HC and ERp57, also need the assistance of CRT for the folding and assembly. Therefore, it was conceivable that the expression of truncated CRT in K42 (CRT-/-) may alter the content of these chaperones in the cells.

To identify any differences in chaperone expression, the levels of calnexin, tapasin, HC and ERp57 from K42 (CRT-/-) cells transfected with truncated CRTs and the parent cell line K42 (CRT-/-) were compared by western blot analysis using serial 2-fold diluted samples of cell lysates (Fig 5.12). This analysis demonstrated that the steady state expression of calnexin, tapasin and ERp57 were all the same for K42-P, K42-PC, K42-NP and K42 cells. However, the overall protein expression level of HC in truncated CRT transfected K42 cells was lower than that of parent K42 cells. The reason for this deference is not clear. As discussed in section 4.2.6, the level of total HC detected by western blot is very sensitive to minor changes in experimental procedure, such as the length of time the cells were digested by trypsin, and even the amount of time the cells were kept in suspension before lysis (unpublished observation). Alternatively, the truncated CRT may affect the protein synthesis of HC and/or its degradation. For example, if the peptide loading onto class I is not efficient, there would be more empty or sub-optimally loaded class I molecules produced, which are subjected to ER associated

degradation by proteasomes. Further investigation is needed to clarify the underlying mechanism.

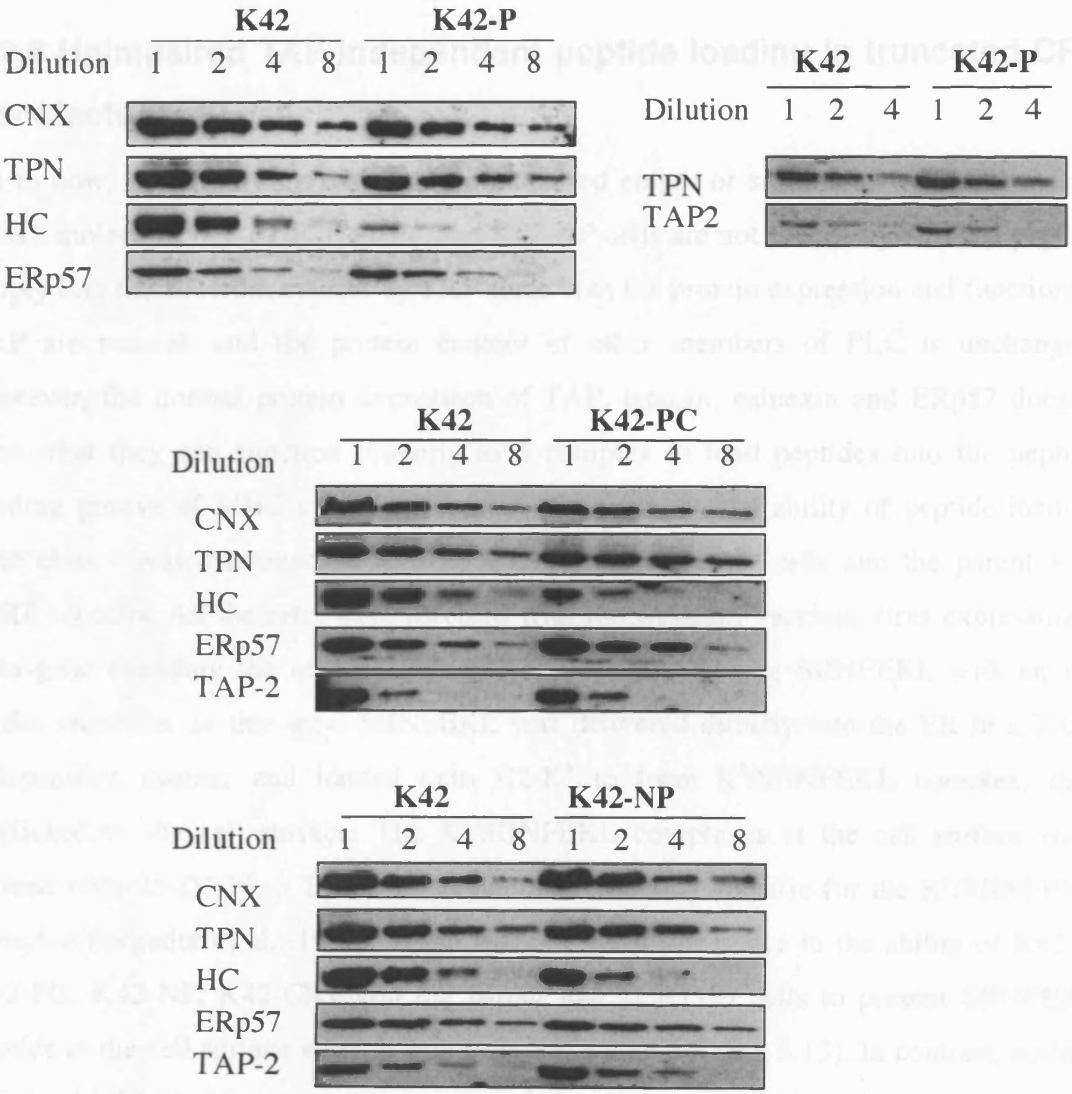


Fig. 5.12 Chaperone content in K42 transfectants

The relative amounts of ER chaperons were analysed by western blot. Two fold serial dilutions of cell lysates for each cell line were subjected to 12% SDS-PAGE and transferred onto Hybond Extra membrane (Amersham), and probed with specific antibodies for calnexin, tapasin, HC, ERp57 and TAP. The results shown are representative of those obtained in three separate experiments.

5.2.6 Unimpaired TAP-independent peptide loading in truncated CRT transfected K42 cells

Up to now, our results suggest that the increased empty or sub-optimally loaded MHC class I molecules in K42-P, K42-PC and K42-NP cells are not caused by reduced peptide supply into the ER from cytosol by TAP since both the protein expression and function of TAP are normal, and the protein content of other members of PLC is unchanged. However, the normal protein expression of TAP, tapasin, calnexin and ERp57 doesn't mean that they can function properly as a complex to load peptides into the peptide binding groove of MHC class I molecules. Accordingly, the ability of peptide loading onto class I was examined in K42-P, K42-PC and K42-NP cells and the parent K42 (CRT-/-) cells. All the cells were infected with recombinant vaccinia virus expressing a mini-gene encoding the mouse class I H-2K^b binding epitope SIINFEKL with an ER leader sequence. In this way, SIINFEKL was delivered directly into the ER in a TAP-independent manner and loaded onto H2-K^b to form K^b/SIINFEKL complex, then trafficked to the cell surface. The K^b/SIINFEKL complexes at the cell surface were stained with 25-D1.16, a TCR-like monoclonal antibody specific for the K^b/SIINFEKL complex(Porgador et al., 1997). There was not much difference in the ability of K42-P, K42-PC, K42-NP, K42-CRT and the parent K42 (CRT-/-) cells to present SIINFEKL peptide at the cell surface when it was delivered to the ER (Fig 5.13). In contrast, surface staining by 25-D1.16 was minimal on cells infected by control vaccinia virus expressing an irrelevant protein, indicating the staining by 25-D1.16 was SIINFEKL specific. Since it is not possible to determination the exact amount of SIINFEKL delivered to the ER during these experiments it is a possibility that a high lumenal abundance of SIINFEKL could compensate for the yet unknown defect in class I peptide loading in K42-P, K42-PC, K42-NP. However, the ability to present ER targeted SIINFEKL means that the truncated CRT proteins in K42-P, K42-PC, K42-NP cells do not cause a global defect in intracellular peptide loading.

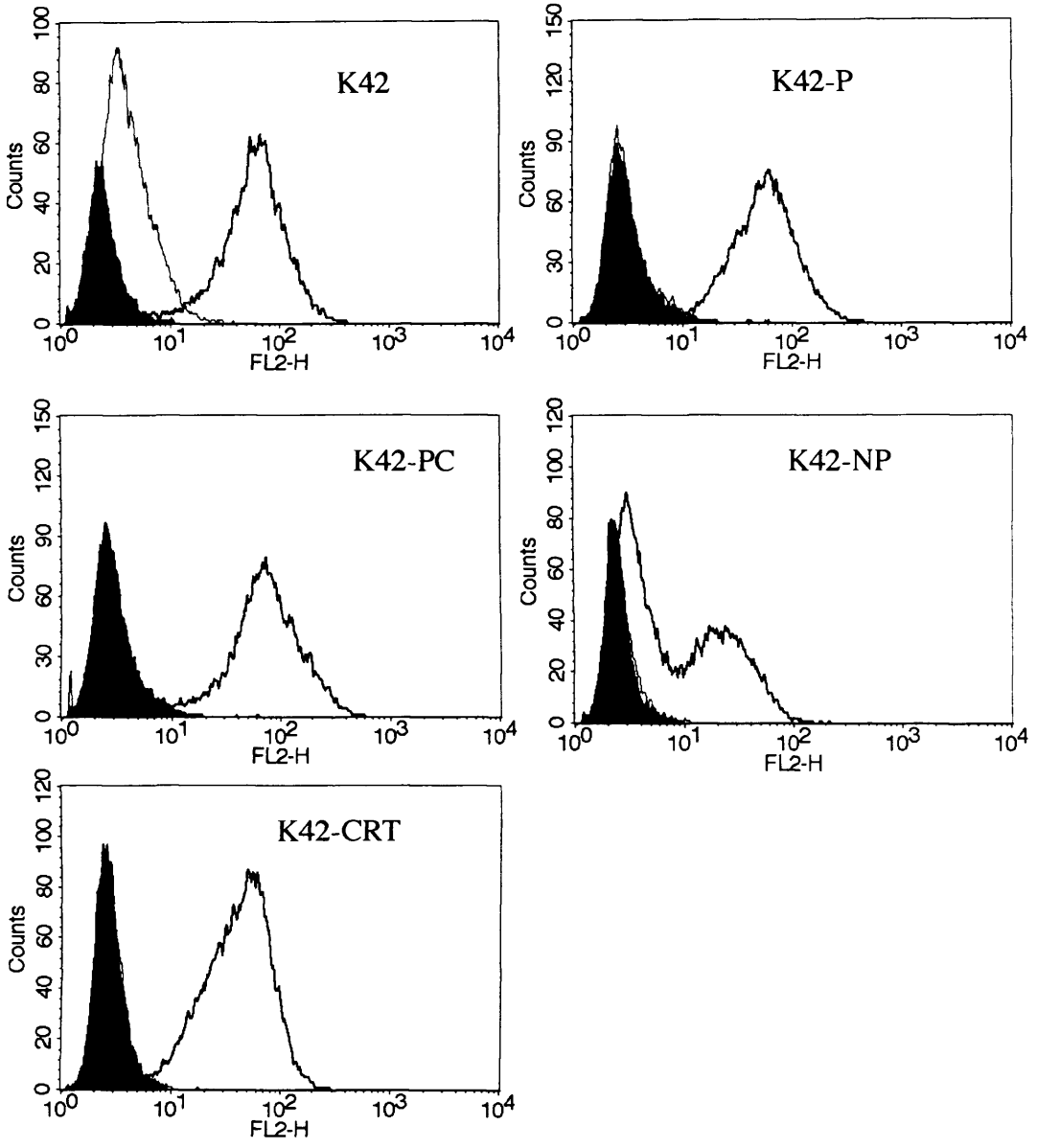


Fig. 5.13 Direct delivery of peptides into the ER rescued surface class I expression

Cells were infected with the same amount of vaccinia virus expressing either SIINFEKL (thick line) with a leader sequence to the ER or expressing irrelevant protein (thin line). Surface MHC class I was analysed by flow cytometry using a TCR-like mAb 25-D1.16, which is specific for SIINFEKL/K^b complex. Shaded peak indicates second antibody only. Data are representative of three independent experiments.

Virus infection rate was appeared to be lower in K42-NP cells than the other cell lines since a proportion of these cells weren't stained by 25-D1.16. This may be caused by the lower number of surface receptors that are responsible for taking in vaccinia virus. This notion is supported by a study showing impaired bradykinin receptor in CRT deficient cells(Nakamura et al., 2001b). They suggested that CRT may play a role in folding of the bradykinin receptors. Nevertheless, the reason behind the low virus infection of K42-NP requires further investigation.

5.2.7 Inefficient class I antigen presentation of cytosolically derived peptides in truncated CRT transfected K42 cells

Transfection of truncated CRT into K42 (CRT-/-) cells resulted in failure to load the majority of class I molecules with high-affinity peptides and then were exported in an unstable state, leading to the reduced cell surface expression of class I. However, as Fig. 5.13 showed, when class I binding peptide SIINFEKL was delivered directly into the ER, K42-P, K42-PC and K42-NP cells can present SIINFEKL in the context of K^b as efficiently as the parental K42 (CRT-/-) cells. To determine the effect of the expression of full length and truncated forms of CRT in K42 (CRT-/-) cells on antigen presentation, their ability to present the ovalbumin derived epitope SIINFEKL to a T cell hybridoma B3Z was assessed(Karttunen et al., 1992; Sanderson and Shastri, 1994). K42 (CRT-/-) cells transfected with truncated CRT presented SIINFEKL derived from cytosolic OVA less efficiently than the parent K42 (CRT-/-) cells (Fig 5.14). K42-P and K42-NP cells were particularly affected. B3Z responses obtained with these cell lines at all OVA concentrations were comparable to cells pulsed with control protein BSA. In contrast, the introduction of CRT into K42 (CRT-/-) cells dramatically increased the efficiency of conventional cytosolic class I antigen presentation. Collectively, these data suggest that CRT fragments in CRT deficient cells have a profoundly negative effect on TAP-dependent peptide loading.

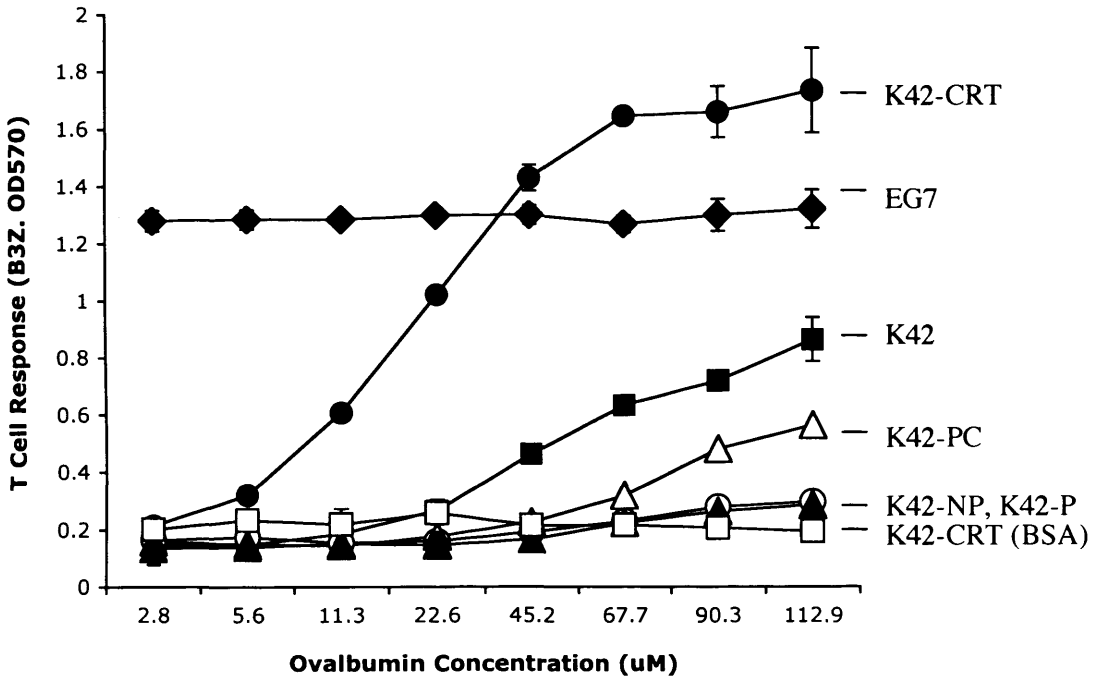


Fig. 5.14 MHC class I presentation of the OVA derived peptide SIINFEKL to B3Z is markedly reduced in truncated CRT K42 transfectants

Streptolysin O permeabilized cells were incubated with different concentrations of ovalbumin, and then co-cultured with the lacZ-inducible, SIINFEKL/K^b-specific B3Z T cell hybridoma overnight. B3Z activation was evaluated by ELISA as described in materials and methods. The experiment was carried out in triplicate at each time and repeated at least three times for each cell line. Data was combined for the plot, and error bars show the SD from the mean.

5.3 Discussion

CRT has been demonstrated to play a central role in the formation of the MHC class I PLC(Sadasivan et al., 1996; Harris et al., 1998; Turnquist et al., 2002) and in MHC class I assembly and its subsequent antigen presentation(Gao et al., 2002). In Chapter 4 it was shown that CRT also maintains the low peptide concentration threshold required in the ER for efficient antigen presentation. To further investigate the functional role CRT plays in the MHC class I antigen presentation and the underlying mechanism, full length human CRT and three separate fragments were cloned: the central P domain, the NP fragment (N+P domains of CRT) and PC fragment (P+C domains of CRT). Using an ER leader sequence these were then targeted into the ER of both CRT deficient K42 cells and CRT competent K41 cells. Expression of full length CRT and its fragments in cells with or without CRT had a different impact on cell surface expression of MHC class I. In K42 (CRT-/-) cells, while reintroduction of full length CRT completely restored the cell surface expression of MHC class I, expression of CRT fragments down-regulated the class I expression at the surface of K42 (CRT-/-). In contrast, all the K41 (CRT+/+) transfectants had the same class I expression level seen in the wild-type K41 (+/+) cells (Fig. 5.7). The results further support the notion that calreticulin is important in MHC class I antigen presentation and also provide some clues to the function of calreticulin. For example, calreticulin might act as a bridge to link other functional components of class I assembly machinery. Alternatively, one domain of calreticulin may be required for interaction with a functional molecule while the other executes another unidentified function. ERp57 could be one such interaction partner in this regard. There are several reports suggesting that the P-domain of calreticulin is responsible for binding to ERp57, which catalyses the formation of disulfide bonds of class I molecules(Farmery et al., 2000; Corbett et al., 1999; Ellgaard and Helenius, 2001).

Calcium storage and homoeostasis are among the important roles that calreticulin plays(Camacho and Lechleiter, 1995; Vassilakos et al., 1998). A change of calcium homeostasis by individual fragments of calreticulin might be one possible reason for the observed changes in surface expression of class I. Kimitoshi et al studied the functions of

individual CRT domains on bradykinin binding and bradykinin-induced Ca^{2+} release(Nakamura et al., 2001b). They demonstrated that bradykinin binding and bradykinin-induced Ca^{2+} release is restored by expression of the N+P domain of CRT. But in this study, the MHC class I expression in CRT deficient cells could not be restored by either P domain or N+P domain of CRT. Instead all three domains of CRT were required to restore the surface expression level of class I on CRT deficient K42 cells . It is not surprising that the C-domain of CRT alone cannot rescue the defect in class I expression in K42 (CRT-/-) cells, as this domain has not been reported to have chaperone function. However, the N+P fragment of CRT could not restore the surface class I expression in K42 (CRT-/-) cells either, although previous studies have suggested that both the N-domain and the P-domain of CRT are responsible for its chaperone function. The distinct domains of CRT have been shown to perform specialised functions, but the chaperone function of the N+P domain of CRT are tightly linked to its role in Ca^{2+} homeostasis the C-domain plays(Nakamura et al., 2001b). For example, Corbett and co-workers have showed that the Ca^{2+} concentration in the lumen of the ER affects the structure, conformation and the chaperone function of CRT, as indicated by changes in its sensitivity to protease digestion in the absence or presence of different concentrations of Ca^{2+} (Corbett et al., 2000). Another study from the same group found that Ca^{2+} concentration in the ER regulated the interactions between ER chaperones. CRT was shown to interact with protein disulfide isomerase (PDI) at low Ca^{2+} concentration (below 100 μM), whereas the protein complex dissociates when Ca^{2+} concentration increases above 400 μM. They suggested that Ca^{2+} binding to the C-domain of CRT is responsible for the Ca^{2+} sensitivity of the interaction between the N-domain of CRT with PDI. Furthermore, the association of CRT with another class I accessory molecule, ERp57, was also modulated by Ca^{2+} binding to the C-domain of CRT(Corbett et al., 1999). As a consequence of the changes in these protein-protein interactions, the chaperone function of the protein in assisting class I assembly also changes. Taken together, it seems the chaperone function of either the P domain or the NP fragment of CRT is supported by the function of its C-domain in binding Ca^{2+}. The work presented in this chapter is also consistent with the idea that the chaperone functions carried out by the N and P domains of calreticulin are supported by the C domain, since without the C domain the expression

of either P or NP fragment of CRT in K42 (CRT-/-) cells failed to rescue the deficiency in surface class I expression in the parent cell line.

The cell surface expression of MHC class I on the CRT deficient K42 cells was 25%-30% of the level seen in CRT competent K41(Gao et al., 2002). The data presented here shows that expression of P, PC and NP domains of CRT further reduced surface expression of MHC class I molecules in K42 cells. It was demonstrated that the down-regulation of MHC class I was due to an increase in empty or sub-optimal peptide loaded class I molecules (Fig 5.9), which were not stable at physiological temperature, but could be stabilized by incubation either at low temperature or with exogenously added class I binding peptide. There are a few possible mechanisms by which truncated CRT could affect MHC class I molecules being loaded with high affinity peptide. Firstly, poor peptide supply in the ER due to dysfunction of TAP leads to empty or sub-optimal peptide loaded class I, as demonstrated by the mouse TAP-2 mutant RMA-S cell line (Ljunggren et al., 1990; De Silva et al., 1999) or human TAP mutant T2(Paulsson et al., 2001). It is possible that TAP protein and/or TAP function in truncated CRT transfected K42 (CRT-/-) cells are affected because the CRT fragments cannot perform chaperone functions properly, which leads to the misfolding and misassembly of TAP. However, both the western blot experiment and peptide transport assay do not support this, since the truncated CRT transfected K42 (CRT-/-) cells were shown to have the same level of TAP protein expression (Fig. 5.12) and peptide translocation ability seen in the parental K42 (CRT-/-) cells (Fig. 5.11). Another possible mechanism by which more peptide receptive class I molecules were generated is that the presence of truncated CRT in K42 (CRT-/-) cells may affect the content of other chaperones in the MHC class I loading complex, such as calnexin, tapasin and ERp57, due to the compromised quality control system. Reduced expression of these chaperones could have a negative effect on the class I antigen presentation. Nevertheless, this was proved not to be the case either, since the similar protein expression level of these chaperones in truncated CRT transfected K42 (CRT-/-) cells to that of parental K42 cells was found by semiquantitative western blot (Fig. 5.12). Alternatively, the ability of PLC to load peptide onto class I molecules could affect the generation of peptide receptive class I molecules, which leads to the reduced

surface class I expression. To determine whether loading is impaired, K^b binding peptide SIINFEKL was delivered into the ER by vaccinia virus and the peptide loading ability of K42 (CRT-/-) cells transfected with CRT fragments was compared with that of parent K42 cells by measuring the amount of cell surface K^b/SIINFEKL complex. Surprisingly, direct delivery of SIINFEKL to the ER in a TAP-independent manner rescued the ability of K42-P, K42-PC and K42-NP to present epitopes at the cell surface (Fig. 5.13). As discussed in chapter 4, although direct peptide delivery into the ER rescued the surface class I expression, it still cannot be excluded that the peptide optimisation process is impaired in these cells as the concentration of peptide expressed by vaccinia viruses is high, which could compensate for the defect in peptide loading(Lybarger et al., 2001). Nevertheless, it is reasonable to conclude that CRT deficient cells do not have a global defect in intracellular peptide loading of peptide. Noticeably, the infection efficiency by vaccinia virus for individual cell line was different as indicated by a class I negative population of cells in K42-NP. Since CRT participates in the quality control of both membrane bound or secreted proteins(Molinari et al., 2004) it is possible that the cell surface receptors responsible for taking up virus were affected by the presence of truncated CRT (especially the NP fragment of CRT) in cells without endogenous CRT. Further studies are needed to address this issue.

It is intriguing that truncated CRT transfected K42 (CRT-/-) cells produced more peptide receptive or sub-optimal peptide loaded class I molecules in comparison to the parent K42 cells, although the possible factors relating to the MHC class I assembly, described above, are normal. Nonetheless, the ultimate aim of class I pathway is to display the peptides generated from cytosolic proteins at the cell surface for recognition by CTLs. To assess the ability of the transfectants to present endogenous antigen, the model antigen ovalbumin were delivered into the cytoplasm by streptolysin O, and B3Z responses were used to quantify the relative amount of surface SIINFEKL/K^b complex(Karttunen et al., 1992; Sanderson and Shastri, 1994). Our results showed that at the same concentrations of cytosolic ovalbumin, the truncated CRT transfected K42 (CRT-/-) cells presented SIINFEKL/K^b to B3Z less efficiently than the parental K42 (CRT-/-) cells. In contrast, expression of full length CRT in K42 cells increased the efficiency of presenting

SIINFEKL in the context of K^b to T cells (Fig. 5.14). These data revealed that the expression of truncated CRT dramatically reduced the ability of K42 (CRT-/-) cells to present endogenous antigen, while full length CRT increased the efficiency.

The antigen-presentation phenotype of K42 (CRT-/-) cells transfected with truncated CRT, described above, was reminiscent of tapasin deficient .220 cells(Lehner et al., 1998) or T134K cell line (threonine at position 134 of HLA-A2.1 HC is changed to lysine)(Peace-Brewer et al., 1996; Lewis and Elliott, 1998; Lewis et al., 1996). T134K and .220 both express lower levels of MHC class I at the cell surface and fail to present endogenously synthesized peptide to CTL, which can be overcome by direct delivery of peptide to the ER using an ER signal sequence. Coimmunoprecipitation experiments showed that MHC class I molecules in these two cell lines do not associate with TAP complex(Peace-Brewer et al., 1996; Lewis and Elliott, 1998; Lewis et al., 1996; Grandea, III et al., 1995; Greenwood et al., 1994), indicating the disruption of MHC class I loading complex. Accordingly, it is possible that the expression of truncated CRT in K42 (CRT-/-) cells affects the formation of the PLC resulting in the phenotype described above. Since in CRT deficient K42 cells, MHC class I is still incorporated into the PLC, although with lower efficiency. It is possible that an unknown redundant protein, such as calnexin, may compensate for the loss of CRT function in K42 cells. Calnexin has been found to be part of the PLC in mouse cells(Gao et al., 2002), but not in human cells where there is no interaction between calnexin and HC/β2m heterodimers. In contrast TAP associates only with class I-β2m dimers and not with free HCs(Ortmann et al., 1994; Nossner and Parham, 1995). Therefore, there may be species differences in how calnexin functions in peptide loading or interaction with the TAP peptide transporter. In murine cells, calnexin may have a more redundant function with CRT in facilitating the peptide loading onto MHC class I molecules. One possible mechanism by which truncated CRT could down-regulate class I molecules in K42 (RT-/-) cells is via a dominant negative effect by competing with calnexin for the formation of a similar multi-protein complex in transfected K42 (CRT-/-) cells. The resulting complex, containing truncated CRT and not the redundant calnexin protein, would be less efficient at loading peptide onto MHC class

I molecules, thus resulting in increased peptide receptive class I, and subsequently reduced class I surface expression. Another possibility is that the expression of truncated CRT in K42 (CRT-/-) cells could simply disrupt the interaction of proteins in the PLC. To test these hypotheses, coimmunoprecipitation experiments were performed to measure the protein-protein interactions in PLC of K41, K42 and their CRT P domain transfectants. Preliminary Results showed that more calnexin was recruited into the PLC of K42 cells compared with that in K41 cells, supporting the idea that calnexin may compensate for the loss of CRT function in the absence of CRT (Data not shown). Despite the reduced surface class I expression on K42-P cells however, the same amount of class I was found in the PLC of K42-P and its parental K42 cells, suggesting that the presence of the CRT P-domain did not affect the formation of PLC. Thus, in the absence of CRT, truncated CRTs, which all contain the central proline rich P domain capable of interacting with ERp57 and also possessing the lectin site of CRT capable of binding oligosaccharide on class I HC(Vassilakos et al., 1998), might compete with calnexin for the formation of PLC to block its function and the function of PLC, leading to the further reduced cell surface expression of MHC class I.

Another possible mechanism by which truncated CRT could down-regulate class I molecules in K42 (CRT-/-) cells is that truncated CRTs may compete with calnexin at an early stage of biogenesis of MHC class I molecules to affect its assembly. As described before, CNX interacts with MHC class I HC upon its co-translational insertion in the ER membrane to facilitate the folding of HC and stabilize it from aggregation as well as promote its assembly with β2m(David et al., 1993; Hochstenbach et al., 1992; Degen et al., 1992; Degen and Williams, 1991). When the interaction of calnexin with class I HCs was prevented by treatment with the oligosaccharide processing inhibitor castanospermine, the HCs underwent a rapid degradation compared with the control cells(Moore and Spiro, 1993). Moreover, Vassilakos et al have demonstrated that in the absence of calnexin interaction, the assembly of class I HCs with β2m was substantially impaired due to misfolding of the HCs(Vassilakos et al., 1996). Transfected CRT fragments, containing the central P-domian with the lectin binding site, could bind the

glycan moieties on class I HC preventing HC interaction with calnexin, which could result in the accelerated intracellular degradation of the HCs without the chaperone function of calnexin. Indeed, our semiquantitative western blot result showed that the steady-state protein expression level of HCs in truncated CRT trasfected K42 cells was lower than that in its parental K42 cells (Fig. 5.12), indicating that the P-domain of CRT may compete with calnexin for binding to free class I HCs to block its function of facilitating the folding of HCs. In this way, the P-domain of CRT further reduced the cell surface class I expression on K42 cells. Furthermore, preliminary result of pulse-chase experiment indicated that there seemed to be less fully Y3 reactive molecules in K42-P cells, which might suggest less fully assembled K_b molecules in the ER.

It could also be argued however that calnexin may not be important in the prevention of intracellular degradation of the HCs as the class I assembly, transport, and cell surface expression is unaffected in CNX deficient cells(Sadasivan et al., 1995; Scott and Dawson, 1995; Prasad et al., 1998). The possible reason for the normal phenotype of calnexin deficient cells is that in the chronic absence of calnexin, alternative chaperones can compensate for the loss of calnexin function. As mentioned in the previous chapter, immunoglobulin binding protein (BiP) has been proposed to be a possible redundant protein to assist the class I HC folding and its assembly with β2m in human cells without calnexin(Nossner and Parham, 1995). One study has demonstrated that prolonged treatment of cells with castanospermine results in increased expression of BiP mRNA. Another study showed that the BiP protein level is upregulated in calnexin deficient cells(Molinari et al., 2004). Therefore, BiP might have a redundant function with calnexin to promote the folding of class I HCs and its assembly with β2m. After the formation MHC class I heterodimers, CRT might compensate for the calnexin function since CRT associates with MHC class I molecules only after HC is assembled with β2m(Sadasivan et al., 1996; Solheim et al., 1997). The redundant function from BiP and/or CRT may explain why the class I assembly, transport, and cell surface expression is unaffected in human CNX deficient cells. However, the association of BiP with free mouse HC has not yet been identified in mouse cells(Nossner and Parham, 1995;

Paulsson et al., 2001). Therefore, with the competition from transfected CRT fragments for binding oligosaccharide on class I HCs, calnexin may be forced out of function. Without the redundant functions from BiP and CRT, class I HCs in K42 truncated CRT transfectants are susceptible to degradation leading to reduced class I HCs in these cells (Fig. 5.12).

There have been conflicting reports about the lectin binding site of CRT. Leach et al suggested that the primary lectin site of CRT resides within the globular N-domain, with a much weaker secondary site within the P-domain(Leach et al., 2002). Whereas, a study done by Vassilakos et al indicated that the P domain of CRT is the lectin site of CRT capable of binding oligosaccharide(Vassilakos et al., 1998). Our data indirectly supports the latter that the P-domain of CRT contains the lectin binding site of CRT.

The data derived from the truncated CRT transfected K42 cells further demonstrated that CRT plays an important role in MHC class I antigen presentation. However, it is unclear why the ER of most eukaryotic species contains both CRT and calnexin; two homologous chaperones with lectin properties. Calnexin is a member of PLC containing CRT, ERp57, tapasin and TAP(Suh et al., 1996; Gao et al., 2002), but its exact function in class I antigen presentation is not clear since class I can fully assembly and transport normally in the absence of calnexin(Scott and Dawson, 1995; Sadasivan et al., 1995). There are reports that calnexin is not required for peptide loading of class I molecules. For example, Sadasivan and colleagues studied the peptide loading of MHC class I molecules and showed that there is no difference in the HOLC profiles of peptides eluted from the calnexin deficient cell line (NKR) or NKR transfected with calnexin cDNA(Sadasivan et al., 1995). Likewise, Prasad et al examined the requirement for calnexin in the assembly of six different class I allomorphs and addressed the quantitative effects of calnexin on specific MHC-peptide complexes. They found calnexin is not required for the efficient assembly of class I molecules with TAP-dependent or independent peptides(Prasad et al., 1998). Nevertheless, it was shown here that calnexin may function as a "backup" chaperone for CRT in the formation of the PLC and peptide loading onto class I

molecules in mouse cells. This may reflect redundancy in the ER quality control. The class I peptide loading process is an adaptation of the general quality control system involving CRT, calnexin and ERp57(Radcliffe et al., 2002), function of calnexin in assisting the formation of PLC and peptide loading, it was also shown in chapter 4, that ERp57 is upregulated in the absence of CRT. Taken together, the phenotype regarding MHC class I assembly and peptide loading in CRT deficient cells and its CRT P-domain transfectants further demonstrated the fidelity of ER general quality control system.

This is the first study to investigate the effect of expression of truncated CRT on class I antigen presentation in a CRT deficient background. The results of these experiments have served to characterize the phenotype of K42 (CRT-/-) cells transfected with CRT fragments, and further demonstrated the importance of CRT in the formation of PLC, class I assembly and antigen presentation. This work also revealed that the very crucial nature of structure-function relationships among the different domains of CRT. Individual domain cannot function properly as a molecular chaperone for class I without the support of other domains.

Another interesting finding was the change in morphological appearance of CRT fragment transfected K42 cells. The morphological appearance of wild-type (K41), calreticulin-deficient (K42) and full length CRT transfected K42 cells was indistinguishable and typical of fibroblasts. The cell lines all attached firmly to plastic, as though K42 cells grew a bit slower than K41 cells and full length CRT K42 transfectants. K42 transfectants with CRT fragments were more rounder than K41, K42 and K42-CRT cells, and did not attach well onto the solid phase of the culture flasks, suggesting that adherance are abnormal. CRT is a multifunctional protein. In addition to its fuction in chaperoning (glyco)protein and Ca^{2+} homeostasis, CRT also plays a role in modulating cell adhesiveness (Michalak et al., 1999; Johnson et al., 2001). It has been reported that changes in levels of CRT expression affect cell shape and motility. Overexpression of CRT correlates with increased cell adhesiveness, while down-regulation of CRT reduces cell attachment to extracellular substrates, and causes cells to be rounder and smoother

than the control cells(Opas et al., 1996; Leung-Hagesteijn et al., 1994). Moreover, embryonic stem (ES) cells lacking CRT have impaired cell adhesion(Dedhar, 1994). The Ca^{2+} srorage and chaperone functions of CRT are consistent with its location to the ER lumen and with its structure, but it is not clear how it can affect cell adhesiveness at the level of the plasma membrane. A number of different mechenisms have been proposed. Further studies are needed to adrress this issue. Nevertheless, the observations made in this chapter are consistent with the previously observed importance of calreticulin in these functions.

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Appendix

P domain (bold letters) similarity between human and mouse calreticulin

Amino acids sequence for human calreticulin

mllsvplllg llglavaepa vyfkeqfldg dgwtsrwies khksdfgkfv
lssgkfygde ekdkglqtsq darfyalsas fepfsnkgqt lvvqftvkhe
qnidcgggyv klfpnsldqt dmhgdseyni mfgpdicgpg tkkvhvifny
kgknvlinkd irckddefth lytlivrpdn **tyevkidnsq vesgsleddw**
dflppkkikd pdaskpedwd erakiddptd skpedwdkpe hipdpdakkp
edwdeemdge weppviqnpe ykgewkprqi dnpdykgtwi hpeidnpeys
pdpsiyaydn fgvlgldlwq vksgtifdnf litndeayae efgnetwgvt
kaaekqmkdk qdeeqrlkee eedkkrkeee eaedkedded kdedeedeed
keedeeedvp gqakdel

Amino acids sequence for mouse calreticulin

mllsvplllg llglaaadpa iyfkeqfldg dawtnrwves khksdfgkfv
lssgkfygdl ekdkglqtsq darfyalsak fepfsnkgqt lvvqftvkhe
qnidcgggyv klfpsgldqk dmhgdseyni mfgpdicgpg tkkvhvifny
kgknvlinkd irckddefth lytlivrpdn **tyevkidnsq vesgsleddw**
dflppkkikd pdaakpedwd erakiddptd skpedwdkpe hipdpdakkp
edwdeemdge weppviqnpe ykgewkprqi dnpdykgtwi hpeidnpeys
pdaniyayds favlgldlwq vksgtifdnf litndeayae efgnetwgvt
kaaekqmkdk qdeeqrlkee eedkkrkeee eaedkedddd rdededeede
keedeeespg qakdel