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**MISFOLDED AND DIMERIC HLA-B27
MOLECULES: IMPLICATION FOR ANKYLOSING
SPONDYLITIS**

A thesis submitted for the degree of

Doctor of Philosophy

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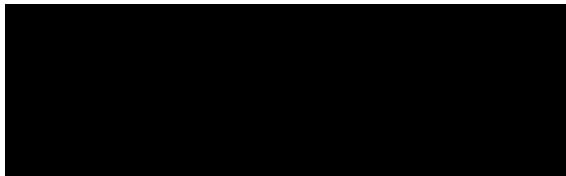
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Declaration

I, Izabela Lenart confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.



Abstract

HLA-B27 is a Major Histocompatibility Complex (MHC) class I molecule which exhibits a strong association with the inflammatory arthritic disorder Ankylosing Spondylitis (AS). It has been postulated that the tendency for HLA-B27 to misfold and form disulphide linked heavy chain homodimers may contribute to AS pathogenesis. However, it remains elusive how these aberrant forms occur and to which extent they participate in AS development.

I have analysed the contribution of conserved cysteine residues at positions (p) 101, 164, 203 and 259 and unpaired cysteines at p67, 308 and 325, in the formation of ER resident heavy chain homodimers. I demonstrated that HLA-B27 dimerisation involves a hierarchy of disulphide bonding. Moreover, my study indicates that heavy chain-dimers are composed of multiple species, with each possibly exhibiting varying degrees of folding/unfolding.

I found that HLA-B*27:05 adopts novel *in vivo* conformations within the ER lumen, which have yet to be identified for other HLA alleles. Detection of these conformations depends on cysteine residue exposure to the ER environment what can explain the propensity for heavy chains to misfold.

Finally, I investigated the role of the MHC class I antigen presentation pathway in HLA-B27 dimerisation. I found that HLA-B27 retains its tendency to dimerise despite the presence of β 2m and high affinity peptide and both β 2m-free (unfolded) and β 2m+peptide-associated (folded) homodimers can associate with the Peptide Loading Complex. Moreover, the tendency for HLA-B27 to dimerise is influenced by p116 within the peptide binding groove, which is a natural polymorphism within AS-associated and non-AS-associated HLA-B27 subtypes.

These studies may have implications for inflammatory disease since they begin to provide a biochemical understanding for the ability of HLA-B27 to misfold.

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Abbreviations

ABC	ATP-binding cassette
AD	Activation domain
AD	Alzheimer's disease
ADP	Adenozyne-5'-difosforan
AMS	4-acetamido-4'maleimidylstibene-2,2'disulfonic acid
ANKENT	Ankylosing enthesopathy
APP	Amyloid precursor protein
APS	Ammonium persulfate
AS	Ankylosing spondylitis
ASK1	Apoptosis signal-regulating kinase 1
APC	Antigen Presenting Cell
ATF4	Activating transcription factor 4
ATF6	Activating transcription factor 6
ATP	Adenozyne-5'-trifosforan
β 2m	β 2 microglobulin
B27-Tg	HLA-B27 transgenic
BAK	BCL-2 homologous antagonist/killer
BAP31	B-cell receptor associated protein 31
BAX	BCL-2-associated X protein
BH	Bleomycin hydrolase
BID	BH3-only protein death agonist
BiP	Binding immunoglobulin protein
BMDM	Bone marrow derived macrophage
BSA	Bovine serum albumin
bZIP	Basic leucine zipper
CHOP	CCAAT/Enhancer-binding protein homologous protein
COPII	Coat protein II
CMV	Cytomegalovirus
CNX	Calnexin
CRE	cAMP response element
CRT	Calreticulin

CTL	Cytotoxic T Lymphocyte
DC	Dendritic cell
Der1p	Degradation in the ER protein 1
DMEM	Dulbecco's modified Eagle's medium
DTT	Dithiothreitol
EBV	Epstein Barr Virus
ECL	Enhanced chemiluminescent
EDEM	ER degradation enhancing alpha-mannosidase-like protein
EF1 α	Elongation factor 1-alpha
eIF2 α	Eukaryotic initiation factor 2 alpha
Endo H	Endoglycosidase H
ER	Endoplasmic reticulum
ERAAP	ER amino peptidase associated with antigen presentation
ERAD	Endoplasmic reticulum-associated degradation
ERdj	ER resident J-domain co-chaperone
ERSE	ER stress response element
Ero	Endoplasmic reticulum oxidoreductin
FACS	Flow cytometry/ Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
FRT	Flippase Recombination Target site
GADD34	Growth arrest and DNA damage-inducible 34
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
Glc	Glucose
GlcNAc	N-acetylglucosamine
Gls	Glucosidase
GlyC	Glycophorin C
Grp78	Glucose-regulated protein 78
GSH	Reduced glutathione
GSSG	Oxidised glutathione
HA	Hemagglutinin
HC	Heavy chain

HDJ	Human ER-associated DNAJ
HLA	Human leukocyte antigen
HRP	Horseradish peroxidase
HSP	Heat shock protein
HSV	Herpes simplex virus
IBD	Inflammatory bowel disease
ICE	Intestinal epithelial cell
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IP	Immunoprecipitation
IP3R	Inositol-1,4,5-trisphosphate receptor
IRE1	Inositol-requiring kinase 1
JNK	c-Jun N-terminal kinase
kD	KiloDalton
KIR	Killer immunoglobulin-like receptor
LAMP1	Lysosomal-associated membrane protein 1
LAP	Leucine aminopeptidase
LILR	Leukocyte immunoglobulin-like receptor
LMP	Low molecular mass polypeptide
LPS	Lipopolysaccharide
mAB	Monoclonal antibody
Man	Mannose
mBBr	monobromobimane
MDG1	Microvascular endothelial Differentiation Gene 1
MECL1	Multicatalytic endopeptidase complex 1
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MICA	MHC-class related chain gene A
MIP-1a	Macrophage inflammatory protein-1a
MMTS	Methanethiolsulfonate
mRNA	Messenger ribose nucleic acid

MS	Multiple sclerosis
Mw	Molecular weight
NEM	N-Ethylmaleimide
NF- κ B	Nuclear factor kappa B
NFY	Nuclear factor Y
NHK	Null Hong Kong
NK	Natural killer
NP	Nucleoprotein
NP-40	Nonidet P-40
Nrf2	Nuclear factor (erythroid-derived 2)-like 2
OR	Odd ratio
ORF	Open reading frame
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PDI	Protein disulfide isomerase
PDILT	Protein disulfide isomerase-like protein of the testis
PERK	Pancreatic ER kinase
PFA	Paraformaldehyde
pI	Isoelectric point
PLC	Peptide Loading Complex
pLMP2	Latent membrane protein 2- derived peptide
PMSF	Phenylmethylsulfonyl fluoride
PS	Persenilin
PSA	Puromycin-sensitive aminopeptidase
PsA	Psoriatic spondyloarthritis
PSMB	Proteasome Subunit Beta type
PTP	Permeability transition pore
pVIPR	Vasoactive intestinal peptide type 1 receptor-derived peptide
Q-PCR	Quantitative Polymerase Chain Reaction
RA	Rheumatoid Arthritis
RAGE	Receptor for advanced glycation end products
ReA	Reactive Arthritis

RIDD	Regulated IRE1-dependent mRNA decay
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute medium
S1P	Site-1 protease
SCT	Single Chain Trimer
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide electrophoresis
SFV	Semliki forest virus
SNP	Single-nucleotide polymorphism
SpA	Spondyloarthropathy
SUMO	Small ubiquitin-like modifier protein
TAP	Transporter associated with antigen processing
TCA	Trichloroacetic acid
TCR	T cell Receptor
TEMED	Tetramethylethylenediamine
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TOP	Thimet oligoendopeptidase
TRAF2	TNF-receptor-associated factor 2
TRAM	Translocating chain-Associating Membrane
Trx	Thioredoxin
UGGT	UDP-glucose:glycoprotein glucosyltransferase
UPR	Unfolded protein response
UPRE	Unfolded Protein Response Element
UTR	Untranslated Region
WB	Western blot
WT	Wild-type
XBP1	X-box binding protein

1 General Introduction

1.1 MHC proteins

The human Major Histocompatibility Complex (MHC), referred to as the Human Leukocyte Antigen (HLA), is a group of genes on chromosome 6 encoding glycoproteins that control cell-to-cell interactions and regulate immune responses. MHC molecules are categorised into MHC class I, class II and class III. The A, B, C, E, F, and G genes belong to MHC class I, whilst the five D genes belong to MHC class II. The classical HLA loci is extremely polymorphic since there are currently 9,154 HLA and related alleles. Within over 7000 of MHC class I alleles [there are](#) more than 2200 HLA-A alleles, nearly 3000 HLA-B alleles and more than 1700 HLA-C alleles_(HLA Nomenclature 2013). In the mouse, the MHC is referred to as histocompatibility 2 (H-2) complex and is encoded on chromosome 17. Although the gene arrangement is somewhat different, similarly to the human MHC, they are organized into regions encoding three classes of molecules.

MHC class I

MHC class I molecules are transmembrane glycoproteins expressed on the cell surface of most nucleated mammalian cells. MHC class I occurs as a heavy chain associated with the light chain β 2-microglobulin (β 2m) encoded on human chromosome 15. Principally, MHC class I molecules present endogenously derived antigens from viruses or intracellular bacteria but can also present

exogenous antigens via a process referred to as cross-presentation. MHC class I-peptide complexes are monitored by immune effector cells such as Natural Killer (NK) cells and CD8⁺ cytotoxic T lymphocytes (CTL) cells belonging to the innate and acquire immune systems respectively.

Whilst, the classical MHC class I molecules, HLA-A, -B and -C are highly polymorphic, the non-classical MHC class I molecules are characterised by both limited polymorphism and expression patterns and present antigens which reflect their specialised function in term of immune stimulatory properties. Non-classical MHC molecules can be subdivided into three groups: HLA-E, -F, -G, and ten MHC class I pseudogenes can be distinguished (HLA-H, -J, -K, -L, -P, -T, -U, -W, -X and -V). In the mouse, MHC class I molecules, are encoded by H-2L, D, and K loci that, unlike their human MHC class I counterparts, are separated by the class II and class III genes. The non-classical murine MHC class I molecules are encoded by H2-Q, T and M loci.

MHC class II

MHC class II molecules are expressed mainly at the cell surface of specialised antigen presenting cells (APCs) such as dendritic cells (DC), macrophages and B cells as well as some endothelial cells and the epithelium of the thymus. MHC class II molecules are composed of an α and β chain, each having two domains— α 1 and α 2 and β 1 and β 2. Since two heavy chains contribute to the peptide-binding groove and both can be polymorphic, the MHC class II polymorphism is more complex than class I. The 12-14 amino acids in length peptides presented by MHC class II molecules are derived from extracellular

proteins and are presented to CD4⁺ T helper cells that can modulate both T and B cell responses by providing signals through cytokine release and/or combination of cell to cell interactions. In comparison to classical MHC class II genes (DR, DP, DQ, in humans and H-2A and H-2E in mouse) the group of non-classical MHC class II molecules (in human DM and DO, in mouse M and O) are involved in regulation of peptide loading onto classical MHC class II molecules.

In addition, the MHC class II region contain genes for Transporter Associated with antigen Processing (TAP) 1 and TAP2 - genes for the active transport of peptides from the cytoplasm into the ER and the Proteasome Subunit Beta type PSMB9 and -8 belonging to the proteasome complex (previously known as Low Molecular mass Polypeptide (LMP) – 2 and 7 respectively).

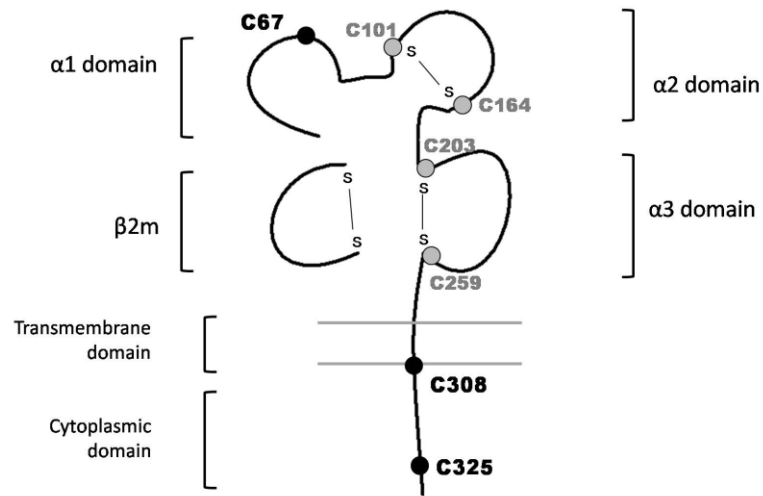
MHC class III

The MHC class III region contains 57–60 structural genes encoding diverse group of molecules. Genes within this region encode some molecules with important immune functions such as the complement system which include components including C4, factor B (BF) and C2, which encode subunit proteins for the C3 and C5 convertases that are crucial for the complement activation pathways of the humoral immune response. The other genes located at the telomeric end of the class III region, encode cytokines such as TNF- α , heat shock proteins, the Receptor for Advanced Glycation End products (RAGE) and others with no known immune function.

1.1.1 MHC class I molecule Structure

MHC class I molecules consist of a Heavy Chain (HC) non-covalently associated with the light chain β 2m. The HC (approx. 45 kD molecular weight (Mw)) is composed of an extracellular, transmembrane and cytoplasmic domain (Figure 1.1A). The extracellular region is composed of 3 domains, α 1, α 2 and α 3. The extracellular α 1 and α 2 domains form the antigen binding groove which bind peptides between 8-10 amino acids in length and are presented to CTLs for recognition via their T Cell Receptor (TCR). The α 3 domain adopts an immunoglobulin like fold and predominantly forms non-covalent associations with β 2m (approx. 12kD) (Bjorkman et al., 1987). The MHC class I tertiary structure is further maintained by two structurally important disulphide bonds between the conserved C101 and C164 residues within the α 2 domain and C203 and C259 within the α 3 domain. Apart from the four conserved cysteine residues shared by all MHC class I molecules, most HLAs possess poorly conserved unpaired cysteines located throughout the heavy chain that do not form intrachain disulphide bonds. Most MHC class I molecules express different numbers of unpaired cysteines: from HLA-G and -A which possess one cysteine, through to HLA-B which possess 2-3 and HLA-C which can have up to 5 cysteine residues. Whilst the majority of HLA-B alleles possess two unpaired cysteine residues either at p67 and p308 or p308 and p325, HLA-B*27:05:02 (further referred to as HLA-B*27:05) expresses cysteines at all three of these positions (Figure 1.1A and B).

A



B

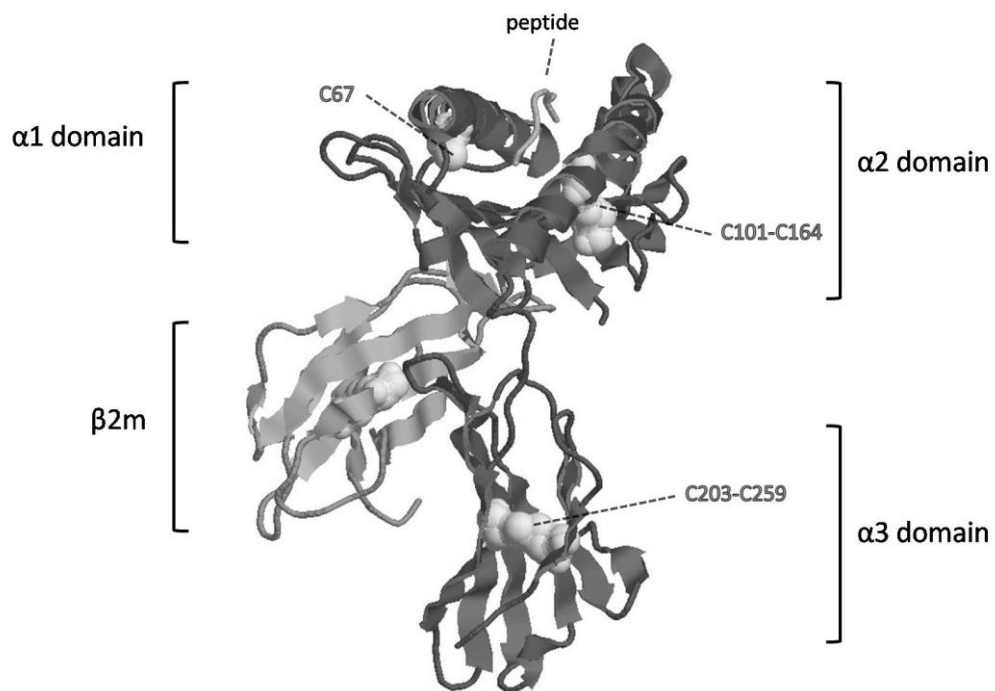


Figure 1.1. MHC class I molecule structure. (A) Schematic structure and (B) ribbon structure of the HLA-B*27:05 molecule with highlighted conserved cysteine residues at position 101, 164, 203 and 259 and unpaired cysteine residues at position 67, 308 and 325 (PDB entry 2BST).

1.2 Protein Folding

In order to be functional, proteins need to acquire appropriate three-dimensional, compact and energetically-favourable structures. Predominantly, protein structure is determined by the sequence of amino acids. The establishment of the hydrogen bonding network between peptide residues leads to adoption of the secondary structural elements such as α -helices and β -sheets. Ultimately, the three dimensional configuration of α -helices and β -sheets, stabilised by hydrophobic interactions, disulphide, hydrogen and ionic bonds constitute protein tertiary structure. Further post-translational modifications such as phosphorylation and glycosylation also contribute to the final protein conformation. The correct protein conformation, called native conformation, is characterised by the most thermodynamically stable state (Dill and Chan, 1997) (Ikai and Tanford, 1971) (Jahn and Radford, 2008) (Tsong et al., 1971).

Although tertiary structure is considered to be largely determined by the protein amino acid sequence, inherent features of polypeptide chains do not ensure the correct assembly. Other determinants of tertiary structure include the environment in which proteins fold, which in the case of MHC class I is the ER lumen, and the presence of chaperones and other accessory molecules (Jonikas et al., 2009).

1.2.1 MHC class I assembly

Like other glycoproteins, the assembly of MHC class I molecules occurs within the lumen of the ER. Folding of MHC class I molecules that occurs via a series of chaperone mediated events can be divided in two stages (Antoniou et al., 2003): (a) early folding events which govern the association of the heavy chain with $\beta 2m$ and (b) later stage characterised by the formation of the Peptide Loading Complex (PLC) .

1.2.1.1 Early events of MHC class I assembly

Calnexin (CNX)

After translocation through the ER Sec61 translocation channel (Johnson and van Waes, 1999), newly synthesized MHC class I HCs associate with $\beta 2m$ (Bjorkman et al., 1987). Newly synthesised heavy chains are cotranslationally attributed with the $\text{Glc}_3\text{Man}_{5-9}\text{GlcNAc}_2$ (Glc, glucose; Man, mannose; GlcNAc, N-acetylglucosamine) glycan attached to the conserved asparagine at p86 within the glycosylation acceptor site Asn-X-Thr/Ser motif of the MHC class I heavy chain (Figure 1.2A) (Abeijon and Hirschberg, 1992). The subsequent trimming of two glucose units by glucosidase I (Gls I) and glucosidase II (Gls II) (Hammond et al., 1994) (Ellgaard and Frickel, 2003) (Lederkremer, 2009) results in the generation of a monoglucosylated unit that is recognised by the ER resident chaperone calnexin (CNX) (Figure 1.2B) (Degen et al., 1992) (Jackson et al., 1994).

CNX is a 88-90 kD transmembrane lectin-like chaperone, which by the sugar binding site located within the β -globular domain, associates with monoglycosylated proteins. Other protein-protein interactions also seem to be involved since mutation of Asn86 does not abrogate the CNX-heavy chain association completely (Harris et al., 1998) (Harris et al., 2001). At the same time CNX via an extended proline rich domain (P domain) recruits the oxidoreductase ERp57 that promotes disulphide bonding within substrate proteins (Oliver et al., 1999) (Pollock et al., 2004). Hence CNX works cooperatively with ERp57 to promote proper folding and disulphide bond formation of newly synthesized MHC class I heavy chains.

When the glycoprotein is completely folded, the terminal glucose is removed by Glc-II and the glycoprotein is released from the CNX-ERp57 cycle (Figure 1.2B) (Hammond et al., 1994) (Lederkremer, 2009). However, if the glycoprotein has not acquired the native conformation, the terminal glucose is once again added by the action of UDP-glucose: glycoprotein glucosyltransferase (UGGT). UGGT is thought to distinguish non natively folded molecules through detection of exposed hydrophobic patches in proximity to N-linked glycans (Sousa and Parodi, 1995) (Labriola et al., 1995) (Caramelo et al., 2003). UGGT-mediated reglucosylation leads to further rounds of lectin binding that promotes constructive folding events. These rounds of deglucosylation/reglucosylation allows the retention of misfolded or partially folded proteins preventing their release to the secretory pathway. As Solda and colleagues proposed, there are some proteins for which UGGT depletion does not affect binding to CNX, hence they complete their folding in one binding event (Solda et al., 2007). For other

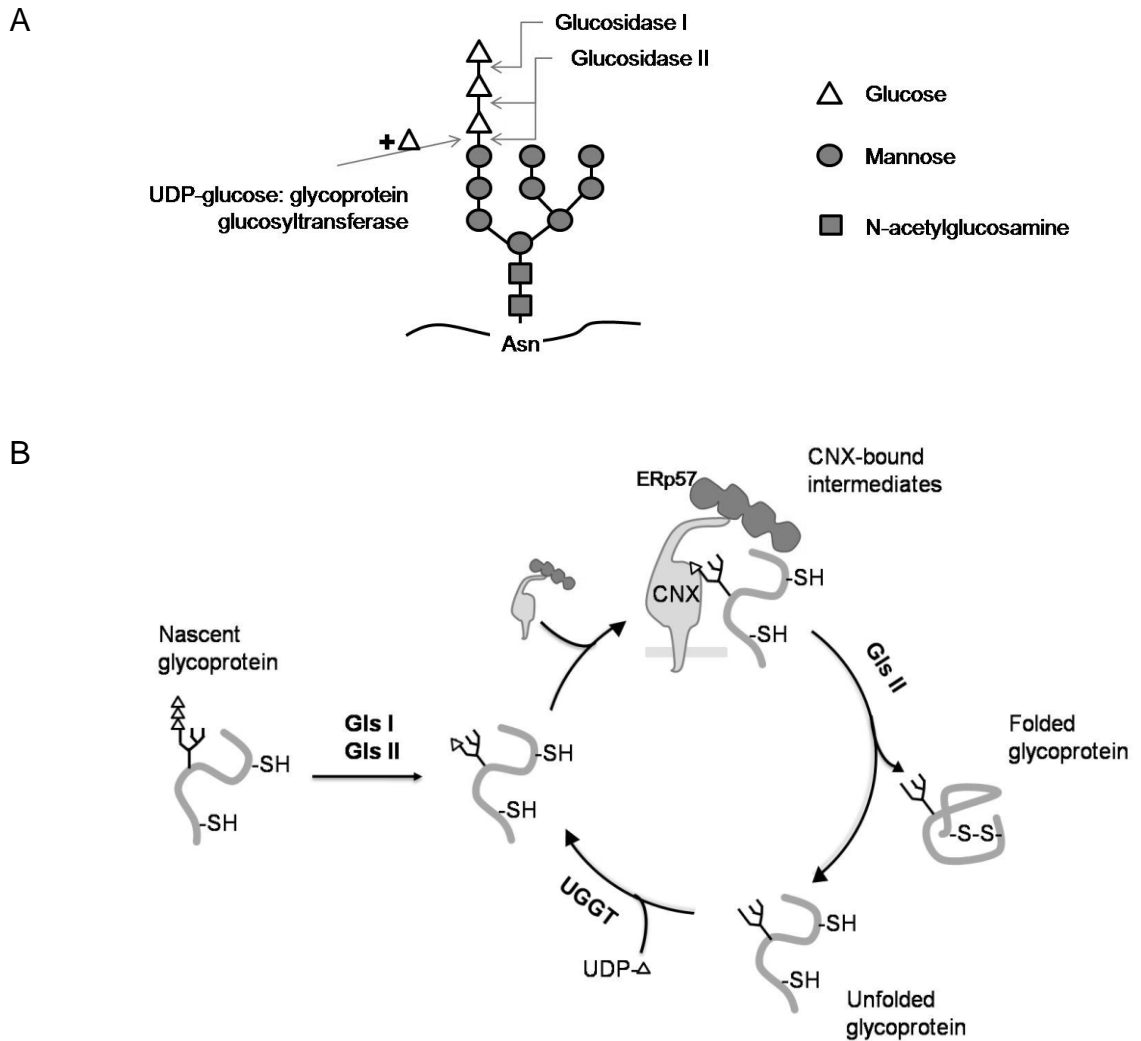


Figure 1.2. The Calnexin cycle. (A) The N-linked core oligosaccharide attached to the asparagines (Asn) residues of the glycoprotein is composed of 2 N-acetylglucosamines, 9 mannoses and 3 glucoses. The terminal glucose can be removed by glucosidase I (Gls I) and the two following glucoses can then be removed by glucosidase II. The terminal glucose residue can be added to the terminal mannose residue, at position I, by UDP-Glc: glycoprotein glucosyltransferase (UGGT). (B) Nascent glycoprotein is cotranslationally attributed with triple-glucosylated core N-linked glycan. The action of Gls I and Gls II generates the monoglucosylated glycan recognized by calnexin (CNX) that recruit the oxidoreductase ERp57 in order to facilitate appropriate disulphide bond formation. Trimming of the final glucose by Gls II releases the substrate from the CNX cycle. However, in case of protein unfolding UGGT can detect exposed hydrophobic patches on the protein surface and allow can reglucosylation of misfolded or partially unfolded proteins leading to their ER retention and reengagement with CNX.

glycoproteins the absence of UGGT-mediated reglucosylation results in their premature release from CNX, indicating that they undergo several binding events. UGGT deletion has been proposed to affect a third class of protein substrates by delaying their release from CNX. This phenotype suggests that their might be a prerequisite for some structural maturation events for this category of proteins to allow for exit from the folding cycle (Solda et al., 2007).

Glycoproteins differ in their CNX dependence for assembly. While genetic absence of CNX impairs maturation of influenza hemagglutinin (HA), there are no gross folding difference of Semliki forest virus (SFV) glycoprotein in CNX-deficient and competent cells (Molinari et al., 2004). MHC class I assembly was found to be facilitated by CNX. Murine CNX prevents aggregation and protects the MHC class I heavy chain from ER-associated degradation (ERAD), a process which targets misfolded proteins of the ER for ubiquitination and subsequent degradation within the cytosol by the proteasome (Degen and Williams, 1991) (Degen et al., 1992) (Jackson et al., 1994) (see Section 1.3.1 *Endoplasmic Reticulum associated degradation (ERAD)*). However, the influence of CNX on human class I molecules is less profound since castanospermine-mediated inhibition of the formation of monoglucosylated units on substrate proteins has more significant effects on mouse than human class I assembly manifested as a dramatic decrease in the assembly of mouse heavy chains with $\beta 2m$ (Vassilakos et al., 1996). Moreover, maturation and transport rates as determined by acquisition of resistance to digestion with EndoH, and cell surface expression of human MHC class I respectively, were found not to be significantly affected in a CNX-negative cell line (Scott and Dawson, 1995)

(Sadasivan et al., 1995). These observations further suggested that CNX function could possibly be compensated for by other chaperones such as immunoglobulin binding protein (BiP) which can also prevent accumulation of unfolded proteins (Stronge et al., 2001). Species related differences in CNX dependence can at least be partially explained by different N-glycosylation pattern/sites, since introduction of a second N-glycan to the human class I molecule A*0201 at p176, a site present in mouse, resulted in increased binding to calnexin and prolonged retention within the ER (Zhang and Salter, 1998). Moreover, whilst human class I heavy chains are released from CNX following association with β 2m, mouse class I molecules can be retained (Suh et al., 1996). These observations suggest that in the murine system, CNX might be required to stabilize the heavy chain- β 2m association until peptide is bound, which is in line with the fact that the affinity between murine MHC class I heavy chain and β 2m is weaker than in human (Hochman et al., 1988).

Calreticulin (CRT)

Following the association of MHC class I heavy chain with β 2m, in human cells CNX is replaced by the lectin-like chaperone, calreticulin (CRT). CRT is a soluble homologue of CNX with similar sugar binding specificity. Similarly to CNX, through the lectin site it binds the monoglucosylated forms of the N-linked core glycans (Parodi, 2000), whilst via a P domain it can associate with ERp57 (Frickel et al., 2002) (Leach et al., 2002).

Despite the overlapping set of substrate glycoproteins, CRT seems to bind a more narrow set of proteins than CNX (Pipe et al., 1998) (Otteken and Moss,

1996) (Peterson et al., 1995) (van Leeuwen and Kearse, 1996) (Keller et al., 1998). The differences in substrate repertoire can be at least partially explained by solubility of CRT, since calnexin expressed as a soluble ER protein was found to bind a similar spectrum of proteins to that of CRT (Danilczyk et al., 2000). From the perspective of MHC class I assembly, CRT is essential during both the early and late stage of folding. CRT binds to the heavy chain-peptide complex prior to peptide binding (Wearsch et al., 2004) and is also a crucial component of the PLC. CRT appears to be necessary for optimal peptide acquisition since recruitment of class I heavy chain to the PLC was impaired in the absence of CRT (Liu et al., 2010) (Del Cid et al., 2010) (Wearsch et al., 2011). Moreover, loading of MHC class I with optimal peptides within the PLC inside the ER was defective in CRT-deficient cells which subsequently resulted in unstable heavy chain-peptide complexes at the cell surface and impaired T cell recognition (Gao et al., 2002) (Howe et al., 2009).

Interestingly, CRT was found to co-purified preferentially with unfolded/misfolded HLA-B27 molecules (Colbert et al., 2010). However as a part of the quality control system CRT, was found not to prevent the export of sub-optimally loaded MHC class I heavy chains but instead promote the retrieval from the Golgi back to the ER via its own KDEL retrieval sequence (Howe et al., 2009). The fate of the retrieved heavy chain and that of HLA-B27 remains elusive but might be either destined for degradation or undergo further rounds of optimal peptide acquisition.

BiP

As already mentioned, BiP also known as glucose-regulated protein 78 (Grp78), assists MHC class I folding during the early steps of assembly (Hendershot, 2004) (Nossner and Parham, 1995) (Fig. 1.3). It is possible that similarly to the CNX/CRT cycle, BiP can act as a protein folding quality system. However, unlike CNX, BiP possibly relies only on monitoring unfolded regions on nascent proteins (Molinari and Helenius, 2000) (Hendershot, 2004).

BiP is a soluble ER homologue of the cytosolic Heat Shock Protein (HSP)70. It binds substrate proteins through a carboxy (C)-terminal domain, whilst the N-terminal nucleotide binding domain, is the binding site for ATP/ADP. When occupied by ATP, BiP exist in an open conformation that has a high on/off rate for polypeptides. The hydrolysis of ATP to ADP, facilitated by the ER resident J-domain co-chaperones (ERdj1-7), results in a closed conformation of BiP, that stabilises the interaction with the substrate protein (Mayer and Bukau, 2005).

BiP was proposed to facilitate both folding and misfolding events within the ER. BiP associates with newly synthesised proteins rapidly but transiently (Hammond and Helenius, 1994). Analysis of the preferred peptide substrate for BiP performed by Flynn et al. (1991) revealed that BiP preferentially recognises linear seven residue long regions of the single polypeptide chain that may be especially exposed during translocation and translation. Hence, BiP binding to such a non-native region was proposed to substitute for the hydrophobic interior of a native globular protein (Flynn et al., 1991). Thus, these studies give some indication as to how BiP may recognise and bind to partially folded polypeptides until appropriate folding or degradation can occur . Whilst the association of

misfolded protein with BiP is prolonged and can initiate Unfolded Protein Response (UPR)- signal transduction cascades, which target terminally misfolded protein for proteasomal degradation, how BiP distinguishes between nascent proteins that have not yet folded and those that are misfolded has not been fully determined.

Simultaneous involvement of BiP in both folding as well as degradation seems to be regulated by distinct activity and substrate specificity of the ERdj family members (Maattanen et al., 2010). It was proposed that ERjd3/6 is involved in folding whilst ERjd4/5 target substrates for degradation since their expression was upregulated by ER stress (Shen et al., 2002b) (Cunnea et al., 2003) (Kurisu et al., 2003). Moreover, ERdj5 due to its reductase activity was proposed to reduce unfolded BiP substrates thus facilitating their retrotranslocation (Cunnea et al., 2003) (Hosoda et al., 2003) (Ushioda et al., 2008).

Some glycoproteins, such as influenza HA protein or SFV p62, do not undergo interactions with BiP (Braakman et al., 1991) (Molinari and Helenius, 2000). These proteins are all characterised by N-glycans located close to the N-terminus, whilst the presence of an N-glycan late in the sequence is thought to predispose glycoproteins to associate with BiP (Molinari and Helenius, 2000). Assembly of many glycoproteins, including HLA is regulated by both the BiP and CNX/CRT quality control systems although simultaneous interactions with both has not been detected, suggesting more of a sequential association process (Nossner and Parham, 1995) (Molinari and Helenius, 2000).

BiP interacts with MHC class I heavy chain during the early stages of folding. Interestingly, it was demonstrated that human HLAs expressed in the C1R cell line exhibited a slower assembly rate which intriguingly correlated with stronger associations with BiP. On the contrary, when the same allele, in this case HLA-B7, was expressed within an EBV cell line its maturation kinetics was faster and heavy chain-BiP associations could barely be detected (Nossner and Parham, 1995). These findings are in line with the characteristics exhibited by HLA-B27 which was found to possess slow maturation kinetics accompanied by prolonged association with BiP (Antoniou et al., 2004). The unusually strong interaction of BiP with HLA-B27 is most likely an indicator that HLA-B27 is prone to misfolding, which could possibly lead to the induction of ER stress (see section Protein misfolding) (Turner et al., 2005).

1.2.1.2 Late MHC class I assembly events

1.2.1.2.1 Composition of The Peptide Loading Complex

MHC class I heavy chain association with β 2m initiates replacement of CNX for CRT and promotes formation of the PLC (Figure 1.3). The PLC brings together heterodimers of MHC class I heavy chain- β 2m associated with CRT/ERp57 tethered to the transporter associated with antigen processing (TAP) via the MHC class I specific accessory molecule tapasin (Park et al., 2006) (Santos et al., 2007) (Figure 1.3). The main role of the PLC is facilitating optimal binding of peptides, which are translocated into the lumen of the ER by the action of the TAP heterodimer, onto MHC class I heavy chains (Lewis et al., 1998).

MHC- β 2m, Tapasin, TAP, CRT and ERp57 are basic components of the PLC. The association of other molecules with the PLC such as the oxidoreductase PDI has also been reported in some studies (Park et al., 2006) (Santos et al., 2007) whilst other studies have failed to demonstrate such interactions (Peaper et al., 2005). Hence the molecular architecture of the PLC and stoichiometry is still controversial. Early reports postulated a ratio 4:1 of tapasin to TAP however, they were undertaken prior to the use of a thiol-reactive reagent that could preserve tapasin-ERp57 conjugates, such as N-ethylmaleimide (NEM) or methanethiolsulfonate (MMTS). Recently, using a 2D-native gel/SDS-PAGE system Rufer *et al.* examined the composition of the PLC and confirmed that it comprised of one TAP heterodimer, two tapasin molecules each disulphide linked to ERp57 but only one MHC class I heavy chain and one molecule of CRT (Rufer et al., 2007). The latter is in line with the hypothesis that CRT is recruited to the PLC together with MHC class I molecules. In agreement with the findings of Peaper *et al* no PDI was detected (Peaper et al., 2005). The presence of only one MHC class I heavy chain could well be an evolutionary mechanism to cope more efficiently with viral infections.

Interestingly, Rufer *et al* also detected an 'alternative' PLC of a smaller overall Mw than the PLC described above. It was found to be composed of TAP/tapasin associated with ERp57 and CRT but lacking any MHC class I molecules. The presence of CRT within this complex supports the notion of a putative precursor TAP complex that precedes the entry of MHC class I heavy chains (Diedrich et al., 2001). Moreover, ERp57 within this alternative PLC was found not to be disulphide bonded to tapasin. This "free" ERp57 may represent

redox-active recycling of Erp57 after the release of the fully assembled MHC class I heavy chain. Hence it was proposed that this 'alternative' PLC complex most likely represents pre- or post-loading intermediates (Rufer et al., 2007). However, contradictory data exists regarding the presence of a CRT-ERp57 dimer in the PLC (Ireland et al., 2008) (Zhang et al., 2009) (Del Cid et al., 2010) (Liu et al., 2010). According to some studies, recruitment of ERp57 into the PLC was not impaired in a CRT-deficient cell line (Liu et al., 2010). Recently, however, using an *in vitro* assay Wearsch *et al.* have found that CRT, bearing mutations that either disrupt glycan binding or the P domain, result in impaired tapasin-mediated peptide binding on MHC class I, indicating that the interaction between CRT and ERp57 are essential for optimal PLC function in an *in vitro* setting (Wearsch et al., 2011).

Nevertheless, most of the data support the model of the PLC assembly proposed by Diedrich *et al.* They found that the TAP-tapasin association is detectable prior to the ERp57 and tapasin interaction which can further recruit CNX and additional ERp57 (Diedrich et al., 2001). The association of these components was irrespective of the presence of monoglucosylated N-linked MHC class I heavy chain, since the castanospermine-mediated inhibition of glucosidase II did not prevent detection of these molecules. Furthermore, the absence of β 2m results in the lack of recruitment of CRT to this complex (Sadasivan et al., 1996). Hence Diedrich proposed that the first step

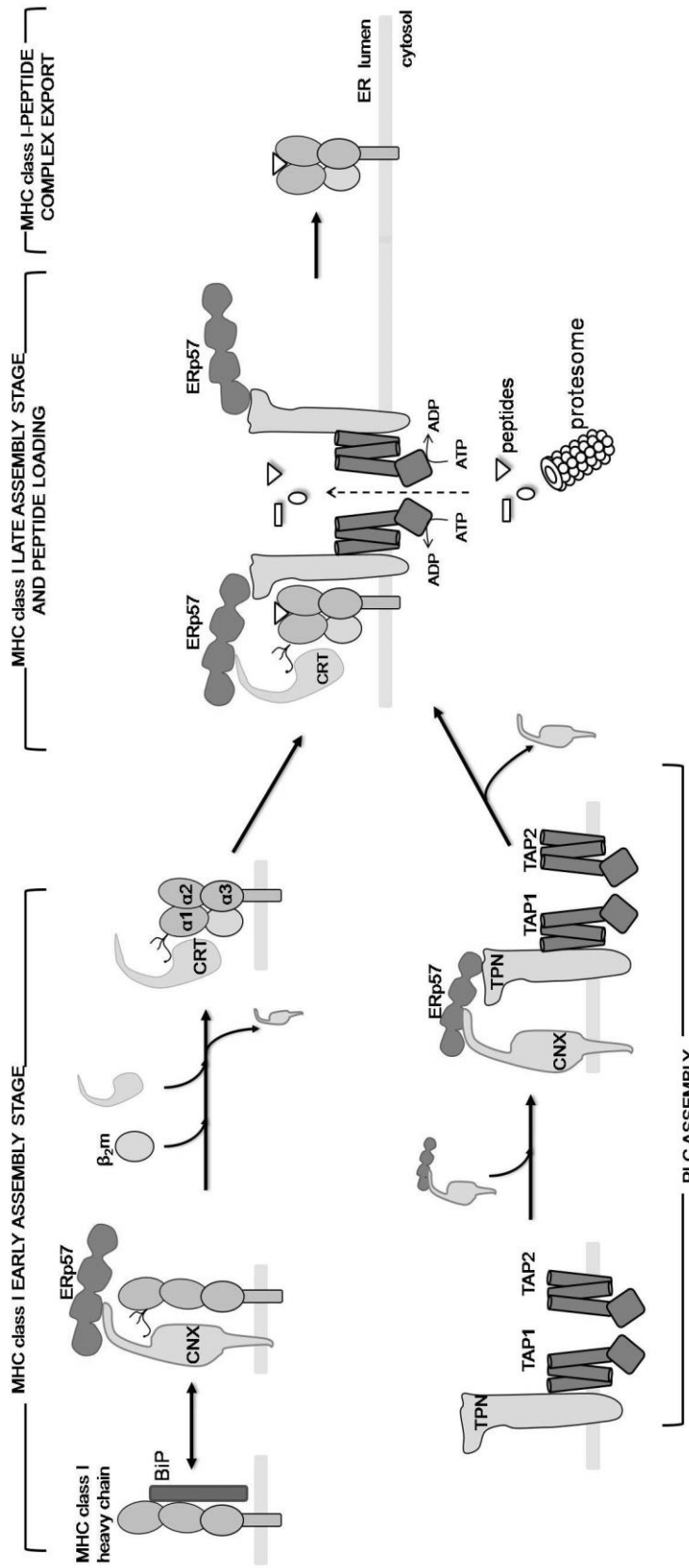


Figure 1.3. Early and late assembly of MHC class I. During the early stage of assembly prior to association with $\beta 2m$ MHC class I heavy chain associates with Calnexin (CNX) that recruits the oxidoreductase ERp57 and/or BiP. Association of the heavy chain with $\beta 2m$ initiates conformational changes that results in the displacement of CNX and the recruitment of Calreticulin (CRT). In parallel, newly synthesized tapasin associates with TAP followed by recruitment of the CNX-ERp57 conjugate. Incorporation of MHC class I heavy chain/ $\beta 2m$ /CRT into this precursor of the PLC is accompanied by the loss of CNX and the acquisition of CRT. The PLC facilitates loading of endogenously-derived peptides that have been translocated into the ER lumen by TAP. Optimal peptide acquisition generates stable MHC class I/peptide complexes that disassemble from the PLC and are transported to the cell surface.

of PLC assembly involves TAP binding to tapasin, followed by the dimer of TAP-tapasin associating with the CNX-ERp57 conjugate. The final stage PLC formation was proposed to involve recruitment of, MHC class I heavy chain- β 2m complexes in associated with CRT. The incorporation of MHC class I is accompanied by the loss of CNX and the acquisition of CRT (Diedrich et al., 2001) (Figure 1.3).

Tapasin

Tapasin is the key MHC class I specific accessory molecule that directs the action of the PLC including successful peptide binding, increasing levels of TAP expression and enhancing peptide transport (Lehner et al., 1998) (Garbi et al., 2003). The discovery of tapasin originated from the observation that the cell line, 220, had a novel defect in HLA class I cell surface expression due to impaired TAP-class I heavy chain association (Greenwood et al., 1994). Later it was discovered that this cell line was deficient in a novel (~48 kD) glycoprotein, named tapasin (Sadasivan et al., 1996) and the role for tapasin as a bridge between TAP and class I heavy chain was proposed. Indeed, tapasin serves as a scaffold for the PLC since it organizes the physical interaction between TAP and MHC class I and tethers accessory chaperones and oxidoreductases.

Structural modelling studies performed by van Hateren *et al.* revealed that there are two potential sites for tapasin-MHC class I heavy chain interactions. These include; (i) the interaction between the tapasin residue R333 and MHC class I residue E222 which is localised within the α 3 domain and (ii) between the tapasin residue R189 and MHC class I T134 which is localised within the α 2

domain (Van Hateren et al., 2010). Furthermore, both tapasin (Sadasivan et al., 1996) and many MHC class I alleles possess unpaired cysteine residues within the transmembrane domain. Direct conjugates of tapasin and MHC class I heavy chains between C420 of tapasin and C308 of MHC class I heavy chain have been reported using an *in vitro* translation system (Chambers et al., 2008), although its physiological relevance has yet to be confirmed.

Tapasin tethers MHC class I heavy chains to TAP, thereby providing the physical proximity of peptide translocation and peptide loading events. Tapasin-TAP interactions occur via the transmembrane domains of both proteins (Koch et al., 2004) (Leonhardt et al., 2005) (Anelli et al., 2002) (Petersen et al., 2005). Each TAP subunit seems to contain a single tapasin docking site within the N-terminal transmembrane helices (Koch et al., 2006), however the sequence motif or residues that mediate the physical interaction have yet to be determined. Tapasin-TAP associations increase the level and stabilise TAP which subsequently enhances peptide transport (Garbi et al., 2003). However the Tapasin-TAP interaction is not essential from the perspective of peptide acquisition since a soluble version of tapasin, which does not associate with TAP, retains the ability to facilitate peptide loading, although full peptide optimisation is not achieved (Lehner et al., 1998) (Tan et al., 2002).

Tapasin was found directly associated with ERp57 via a disulphide bond between the unpaired C95 of tapasin and C57 of ERp57 located within the catalytic N-terminal CXXC motif (Antoniou et al., 2003) (Dick et al., 2002). The recombinant ERp57-tapasin heterodimer was demonstrated to facilitate efficient loading of high-affinity peptides and stabilise peptide-receptive class

I molecules where tapasin alone was ineffective (Wearsch and Cresswell, 2007) (Dick et al., 2002). The presence of oxidoreductases within the PLC suggest that redox regulation is an important process in peptide optimisation which will be discussed later (see section 1.2.2.3.2 Redox regulation within the PLC and during peptide *loading*).

TAP

TAP is responsible for transporting preferentially ~8-16 amino acid long peptide fragments into the ER to be loaded onto MHC class I complexes (Scholz and Tampe, 2009). In TAP-deficient cells, MHC class I molecules are unstable and unable to present cytosolic antigens to CD8⁺ T cells (Van Kaer et al., 1992). TAP is formed of two subunits: TAP1 and TAP2 which together are responsible for the ATP-dependent peptide translocation into the lumen of the ER (Androlewicz et al., 1993) (Neefjes et al., 1993) (Shepherd et al., 1993). When ATP and proteasome generated peptides bind to the TAP complex, they initiate structural rearrangements of TAP that allows for the delivery of peptide ligands into the ER (Gorbulev et al., 2001) (Chen et al., 2003) (Chen et al., 2003). The last residue of the C-terminus and the first three at the N-terminus of peptides are critical for their interaction with TAP (Scholz and Tampe, 2005) and, consistent with the specificity of the MHC class I peptide binding domain, the hydrophobic C-terminal residue interacts more efficiently with TAP (Momburg et al., 1994).

1.2.1.2.2 MHC class I peptide optimisation

Since the main function of MHC class I molecules is to present foreign peptides to cytotoxic T cells, the selection of peptide ligands is towards those which have a longer off-rate providing maximal longevity at the cell surface (Sijts and Pamer, 1997) (Lewis and Elliott, 1998). Tapasin optimises MHC class I peptide binding by exchanging suboptimal, low affinity peptides for those which bind more stably (Lewis and Elliott, 1998) (Williams et al., 2002) (Howarth et al., 2004) (Tan et al., 2002). Alternatively, recent studies indicate that tapasin may act more as a facilitator of peptide binding rather than an editor of the peptide repertoire since the binding affinities of ligands eluted from tapasin-negative and -positive cell lines were equivalent (Zarling et al., 2003). It suggests that tapasin may provide additional stability to the already existing MHC class I-peptide complex.

Not all MHC class I alleles associate with tapasin and require it for peptide editing (Peh et al., 1998). Tapasin independent alleles can optimise their peptide cargo regardless of the PLC (Peh et al., 1998). Much of the work regarding tapasin dependence/independence has been performed with two MHC class I subtypes, HLA-B*44:05 and HLA-B*44:02 in which a single amino acid difference at p116 (aspartic acid in B*44:02 and tyrosine in B*44:05), places them at the opposite ends of the spectrum for the requirement of tapasin for cell surface expression (Leach et al., 2002). Residue 116 lies at the floor of the F pocket and interacts with the side chain of the C-terminal anchor residue of the peptide ligand. Later studies revealed that along with p116 the adjacent p114 residue influences the MHC class I tapasin-dependence phenotype since

a glutamic acid to histidine substitution at p114 within tapasin-dependent B*44:02 allele allows for high-affinity peptide acquisition in the absence of tapasin (Park et al., 2003). The in silico molecular dynamics (MD) studies and experiments revealed that the region around the F pocket of the peptide binding groove of the tapasin-dependent molecule HLA-B*44:02 is in a disordered state when compared to the tapasin-independent HLA-B*44:05 allele (Sieker et al., 2007) (Garstka et al., 2011) and the interaction with tapasin stabilizes the HLA-B*44:02 peptide binding groove to ensure appropriate peptide binding. Hence, tapasin plays a key role in transforming the peptide-binding groove into a high-affinity, peptide-receptive conformation.

Interestingly, different MHC class I alleles display distinct associations with the PLC (Neisig et al., 1996). Precipitation experiments that analysed incorporation of several different alleles into the PLC revealed that the majority of HLA-A and -C alleles tested interacted efficiently with the PLC, whereas a number of HLA-B alleles associated weakly or not at all with TAP (Neisig et al., 1996).

1.2.1.3 The generation of antigenic peptides

Peptide binding

MHC class I molecules preferentially bind 8-10 amino acid long peptide ligands. The peptide repertoire is defined by the polymorphic sequence of the MHC class I peptide binding groove (Madden, 1995) (Elliott et al., 1993) that comprises of six pockets (A-F) which differ in depth, electrostatic potential, and hydrophobicity (Saper et al., 1991). The optimal peptide for the particular HLA has the correct length in order to form maximal number of conserved hydrogen

bonds and correct canonical anchor residues. The side chains of the two anchor residues at position P2 and P Ω (the C-terminal residue), point directly into the floor of the B and F pocket respectively. The D pocket accommodates residue P3 whilst P1, P4 and P8 of nonamers or P9 of decamer does not or exhibit minimal interactions with the peptide binding groove and are responsible for interactions with TCR.

Peptide generation

Peptides presented by MHC class I are typically generated by the proteasome within the cytoplasm (Strehl et al., 2005) (Yewdell and Nicchitta, 2006). Inhibition of proteasome activity can alter the spectrum of peptides bound by MHC class I molecules (Hughes et al., 1996). The proteasome is evolutionarily well conserved and is a large multicatalytic proteolytic complex which is localised within the cytoplasm and the nucleus in eukaryotes (Chen et al., 2011) (Peters et al., 1994). It is a cylindrical complex composed of a 20S "core" of four stacked rings around a central pore. While α subunits that make up the outer two rings are involved in assembly, the β subunits that make the inner two rings participate in the generation of the proteolytic active site. The α subunits bind to the 19S "cap" structures that recognize polyubiquitin attachments on protein substrates and initiate the degradation process. Interestingly, the composition of the 20S core can differ between cell types what can subsequently influence the cleavage site preferences of the proteasome and can potentially result in the production of different peptides across distinct tissues (Kuckelkorn et al., 2002). Using proteasomes from various tissues to generate the epitopes of the HSP60 polypeptide substrate Kuckelkorn *et al.* observed organ-specific preferences in

cleavage pattern which correlated with the outcome of the CTL response (Kuckelkorn et al., 2002). This further raises the idea that the proteasomal system/processing may contribute/account for organ-specific pathology.

Proteasomal cleavage can occur after acidic, hydrophobic or basic residues and results in the generation of peptides that are compatible with the C terminus of the MHC class I antigen binding groove, but possess an extended N terminus with varying lengths (Cascio et al., 2001). Hence, further proteolytic cleavage by aminopeptidases located either within the cytosol or the ER are required to adjust peptides to the required length. Several cytosolic proteases have been identified to trim MHC class I peptide precursors including: leucine aminopeptidase (LAP), bleomycin hydrolase (BH), puromycin-sensitive aminopeptidase (PSA), thimet oligoendopeptidase (TOP) (Beninga et al., 1998) (Stoltze et al., 2000) (Saric et al., 2001) (York et al., 2003), although the latter might predominantly be responsible for epitope degradation (Saric et al., 2001) (York et al., 2003).

It has been proposed that the cytoplasmically located Tripeptidyl Peptidase II (TPPII) can act in concert with the proteasome in the generation of MHC class I peptides. TPPII can cleave >14 amino acids long peptides from the N terminus (Tomkinson, 1999) (Levy et al., 2002) (Seifert et al., 2003). Whether TPPII acts alternatively to or downstream of the proteasome system, remains undetermined.

The main source of MHC class I-associated peptides are misfolded or defective ribosomal products generated by mistranslation from standard open reading

frames (Reits et al., 2000) (Yewdell, 2002). Despite the abundance of defective ribosomal products, antigen presentation is relatively inefficient. Casio et al. show that only 1 out of 10 ovalbumin molecules produce antigenic epitopes *in vitro* (Casio et al., 2001). The majority of peptide fragments reinforce the amino acid pool and hence are lost for immune detection whereas only around 1% of the degradation products are transferred into the lumen of the ER (Fruci et al., 2003) (Reits et al., 2003).

With respect to the immune response, distinguishing self from non-self antigens is essential. During for example a viral infection, the expression of viral gene products is high which probably increases their turnover and likelihood for proteasomal degradation over self-proteins (Yewdell, 2003). Moreover, viral infection is accompanied by up-regulation of IFN- γ , which induces the proteasome regulators PA28 and the formation of immunoproteasomes that both influence antigen processing efficiency (Kloetzel, 2004). The immunoproteasome is a proteasome in which the constitutive catalytically active subunits from the 20S core are replaced by immunosubunits LMP2 (low molecular mass polypeptide 2) (ib1), LMP7 (ib5) and Multicatalytic endopeptidase complex subunit (MECL-1) (ib2). As shown by *in vitro* studies, this replacement can enhance the efficiency of proteasome mediated processing of some antigenic viral peptides (Cerundolo et al., 1995) and alter the cleavage specificity of the core 20S proteasome (Boes et al., 1994) (Gaczynska et al., 1994). However, it was observed that while immunoproteasomes increase presentation of immunodominant epitopes to CTL, they do not or exhibit little influence on the generation of subdominant

epitopes. Furthermore, the immunoproteasome can even negatively affect CTL epitope processing which has been demonstrated for some tumour-associated antigens (van Hall et al., 2000) (Morel et al., 2000).

The peptide precursors generated in the cytosol are subsequently transported across the ER membrane in an ATP-dependent manner by TAP. As already mentioned, these peptides can be up to 16 residues in length (van Endert et al., 1994) (Uebel et al., 1995). Although the C-terminus appears to be fixed, these peptides require final trimming from their N terminus within the ER. A predominant N-terminal trimming activity was found to be mediated by the action of the ER-aminopeptidases ERAAP/ERAP-1 and ERAP-2 (Saric et al., 2002) (York et al., 2002) (Chang et al., 2005) (Saveanu et al., 2005) (Hammer et al., 2006) (Hammer et al., 2007). ERAP1 and ERAP2 are soluble ER enzymes, that shares 49% structural identity (Chang et al., 2005). They are both expressed in human but in mice ERAP2 is present only as a pseudogene. ERAP1 trims oligopeptides from the N-terminus with little sequence specificity, other than poor cleavage of the X-Pro bond (where 'X' is any amino acid), whilst ERAP2 cleaves peptides with basic residues as demonstrated by enzymatic analysis (Serwold et al., 2001) (Saric et al., 2002) (Chang et al., 2005). ERAP1 and 2 can function in a complementary manner and have been demonstrated to colocalise together *in vivo* possibly as heterodimers as suggested by immunoprecipitation experiments (Saveanu et al., 2005) Using synthetic peptides, Chang *et al.* showed that ERAP1 strongly prefers substrates of 9-16 amino acids, which is similar in length to those transported by TAP (Chang et

al., 2005). Peptide trimming appears to stop after the production of minimal nonamer epitopes (Saveanu et al., 2005).

The mechanism of ERAP1 function as a “molecular ruler” was recently confirmed by the ERAP1 crystal structure (Kochan et al., 2011) (Nguyen et al., 2011). This model proposes that the N-terminus of exclusively 9-15aa long peptide precursors was enclosed in the cavity of the ERAP1 active site while the hydrophobic C-terminus was anchored away from the catalytic site but at a distance of around eight or nine residues. Successful cleavage occurs until the peptide can no longer reach the active site from the point where the C-terminus is anchored (Chang et al., 2005). This model also assumes that peptide trimming is only predicted by the aminopeptidase and is independent of MHC class I molecules. However, another study reports the presence of crosstalk between ERAP1 and MHC class I heavy chain since in the absence of MHC class I, recombinant ERAP1 destroys antigenic epitopes in an *in vitro* system (Kanaseki et al., 2006). Hence, whether MHC class I molecules play any role in generating the final antigenic peptide remains a challenge.

The importance of the ERAP system in MHC class I peptide presentation became apparent when the reduced expression of ERAP1 through RNA interference resulted in the impaired trimming of peptides for MHC class I molecules leading to reduced expression of MHC class I molecules at the cell surface and impaired T cell recognition (York et al., 2002) (Serwold et al., 2002). Furthermore, in the absence of ERAP1, mouse cells displayed unstable and structurally unique immunogenic peptide-MHC complexes which were able

to generate potent CD8⁺ T cell and B cell responses, indicating that ERAP1 activity is crucial for proper peptide presentation (Hammer et al., 2007).

The role of ERAP1 in generating stable MHC-peptide complexes is of importance in light of recent genome wide screens which revealed strong genetic associations of ERAP1 with inflammatory diseases including AS (Burton et al., 2007) (Brown, 2008) and psoriasis, a skin disease commonly associated with AS (Strange et al., 2010). How ERAP1 polymorphism is implicated in disease development will be discussed later (see section Non-MHC genes in AS).

A separate question is how TAP-translocated peptides are delivered to the MHC class I peptide binding groove. In the proteolytic environment of the ER free peptides are unstable (Reits et al., 2003) thus it was proposed that delivery of peptides for binding by heavy chain is an active process involving chaperones that protect them from degradation. One proposed candidate to maintain such function is protein disulphide isomerase (PDI). PDI is a multifunctional ER thiol-based oxidoreductase. While its two terminally located active domains are directly involved in the formation and breakage of disulphide bonds, the centrally located non-catalytic b' domain provides the main site for binding substrate or peptides (Cheung and Churchich, 1999) (Klappa et al., 1998a). Recently, PDI was found to associate with the PLC (Park et al., 2006). Using radio-labeled peptides, PDI was identified as a molecule which binds TAP-translocated antigens with high efficiency (Lammert et al., 1997) (Spee and Neefjes, 1997). Moreover, this interaction seems to be specific since functional inhibition of the substrate binding domain within PDI abrogates PDI-

peptide association (Lammert et al., 1997). Hence, PDI was proposed to be the main carrier for TAP-translocated peptides. How does PDI bind and release peptides remains elusive however, it was proposed that it occurs in a redox-dependent manner which will be discussed later (see section 1.2.2.3.3 Redox-regulated MHC class I export).

HLA-B27 displays several unusual characteristics regarding peptide binding. Firstly, it was found to bind longer peptides (Urban et al., 1994). Using the monoclonal antibody MARB4, Urban *et al.* purified a small (5-10%) HLA-B27 population that bound peptide between 8-33aa long whilst using the conformationally dependent antibody ME-1 reactive B27 population, these acquired canonical 8-12aa long peptides (Urban et al., 1994). However, the authors were not able to isolate an individual long peptide due to a low total yield of peptides and the physiological meaning of the binding and presentation of longer peptides in context of HLA-B27 is elusive. However, it was observed that extended peptides can sensitize antigen presenting cells to lysis by MHC class I-restricted CTLs (Gotch et al., 1987) (Robbins et al., 1989). Moreover, peptides longer than nonamers have increased off-rates hence making the heavy chain less stable (Parker et al., 1992) (Cerundolo et al., 1991). Further characterisation of the cell surface MARB4-reactive pool of HLA-B*27:05 molecules, revealed that although associated with peptide (canonical) it represented B27 β 2m-free heavy chains (Malik et al., 2002).

The other unusual characteristics of HLA-B27 is its ability to bind some self-peptides in two distinct conformations (Wucherpfennig, 2004). Crystallographic data revealed that HLA-B*27:05 exclusively, but not the non-AS associated

B*27:09 allele, can present pVIPR (vasoactive intestinal peptide type 1 receptor) derived peptide in two conformations: conventional, in which P5 of the peptide was solvent exposed and thus available for TCR recognition, and a second conformation, in which Arg5 formed a salt bridge with a buried polymorphic residue at p116 located at the floor of the F pocket of the peptide binding groove. This subsequently influenced the positioning of P3-P7 and the overall peptide conformation (Wucherpfennig, 2004). Although it was estimated that only a small subset of B27-restricted peptides would adopt this alternative conformation it was found to influence T cell receptor recognition (Hulsmeyer et al., 2004) (Wucherpfennig, 2004). Moreover despite pVIPR being presented by both B*27:05 and B*27:09 subtypes, the pVIPR-restricted T cell repertoire is much more abundant in B*27:05-positive healthy individuals and AS patients (Fiorillo et al., 2000). Although, the antigen conformation was previously found to influence immunogenicity (Tynan et al., 2005), the physiological implication of a dual peptide conformation in AS pathogenesis is questionable and will be discussed later.

1.2.1.4 MHC class I complex disassembly

Optimal peptide stably binds to the heavy chain and increases the affinity between heavy chain and β 2m subunit (Elliott et al., 1997). It also can induce conformational changes in the overall MHC class I structure (Elliott et al., 1997) (Springer et al., 1998) (Smith et al., 1993) (Yu et al., 1999) (Zacharias and Springer, 2004). The latter is supported by observations that conformation-sensitive monoclonal antibodies only detect peptide-loaded MHC class I

molecules (Yu et al., 1999). Furthermore, in comparison with peptide-occupied MHC class I, the peptide receptive conformation was proposed to possess many of the properties ascribed to the molten globule state (Bouvier and Wiley, 1998).

However, recently it has become evident that optimal peptide binding is not sufficient to trigger MHC class I release from the PLC and egress from the ER. Other factors include detachment of MHC class I-peptide complexes from the TAP heterodimer. Knittler and colleagues reported that disruption of the TAP nucleotide binding site and nucleotide-depletion prevents release of properly loaded MHC class I heavy chains from the PLC (Knittler et al., 1999). Hence, conformational signals transmitted from TAP may be necessary for heavy chain dissociation. How the nucleotide binding cycle regulates both translocation of peptide precursors by TAP and MHC class I release remains elusive. This could be analogous to the mechanism observed in the case of the ATP-binding cassette (ABC) transporter P glycoprotein in which nucleotide binding, but not hydrolysis, drives the large conformational changes (Rosenberg et al., 2001). Furthermore, MHC class I disassembly was found to be dependent on PDI-mediated dissociation of the tapasin-ERp57 heterodimer. That will be discussed in the chapter dedicated to redox regulation during the MHC class I assembly (see section Redox-regulated MHC class I export).

Furthermore, until recently it was considered that the removal of the final glucose unit from the MHC class I heavy chain was a signal for release from the PLC (van Leeuwen and Kearse, 1996). However, new data supports the model in which MHC class I heavy chains undergo the final GlcII-mediated

deglycosylation after release from the PLC (Wearsch et al., 2011). Hence potentially, repeated peptide loading events can take place until the most optimal (highest affinity) peptide is acquired (Wearsch et al., 2011). Together with the finding that UGGT can recognise heavy chain loaded with suboptimal peptide suggests that the MHC class I glycosylation state can play a role in peptide exchange and optimisation (Wearsch et al., 2011) (Zhang et al., 2011).

MHC class I Complex Exit

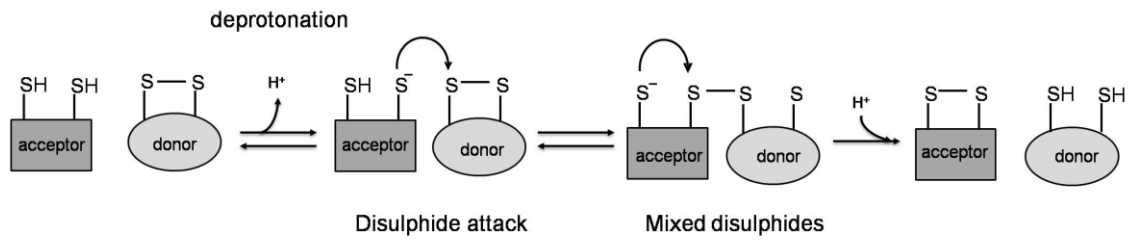
The export of peptide-loaded MHC class I molecules is a selective transport mechanism. Upon release from the PLC, peptide-loaded MHC class I molecules have been proposed to cluster at ER exit sites where COPII coat protein complexes mediate curvature of the ER membrane and formation of transport vesicles. The COPII-coated vesicles transfer MHC class I to the Golgi apparatus via the ER Golgi intermediate compartment. The recruitment of MHC class I to the ER exit site was proposed to be triggered through a putative cargo receptor B-cell receptor associated protein 31 (BAP31) (Spiliotis et al., 2000) (Paquet et al., 2004) (Abe et al., 2009) (Ladasky et al., 2006). BAP31 is a transmembrane receptor which was shown to increase export of MHC class I molecules in a dose dependent manner (Ladasky et al., 2006). Moreover, in the absence of BAP31 the exit of MHC class I molecules was delayed and colocalisation with components of the COPII complex impaired (Paquet et al., 2004). However, BAP31 depletion does not affect MHC class I cell surface expression suggesting that its involvement might be redundant (Ladasky et al., 2006).

Remarkably MHC class I can egress from the ER irrespective of optimal peptide binding (Garstka et al., 2007). Analysing purified COP-II vesicles Garstka et al. show that both peptide loaded and peptide-receptive MHC class I heavy chain can exit the ER and traffic to the cis-Golgi compartment. However, only MHC class I loaded with high affinity peptide is shuttled further to the cell surface (Garstka et al., 2007). Peptide-receptive or suboptimal loaded MHC class I molecules are thought to cycle between ER and cis-Golgi and it was suggested that CRT was responsible for its retrieval since these two molecules co-localise to the ER-Golgi intermediate and cis-Golgi compartment (Howe et al., 2009). Moreover, other components of the PLC such as tapasin were found to participate in MHC class I retrieval (Paulsson et al., 2002). By mutating a double lysine motif within the sequence of tapasin, Paulsson *et al.* (2002) demonstrated a role in retrograde transport of immature MHC class I molecules to the ER (Paulsson et al., 2002) (Paulsson et al., 2006). While retrieval of MHC class I molecules back to the ER would allow for further rounds of peptide editing until optimal peptide is acquired, some evidence suggests that peptide optimisation can also occur in post-ER compartments (Williams et al., 2002).

1.2.2 Protein Oxidative Folding

The formation of native disulphide bonds is an essential event in the folding of proteins entering the secretory pathway. Disulphide bonds are unique among protein interactions as they are the most stable thus their influence on protein

A



B

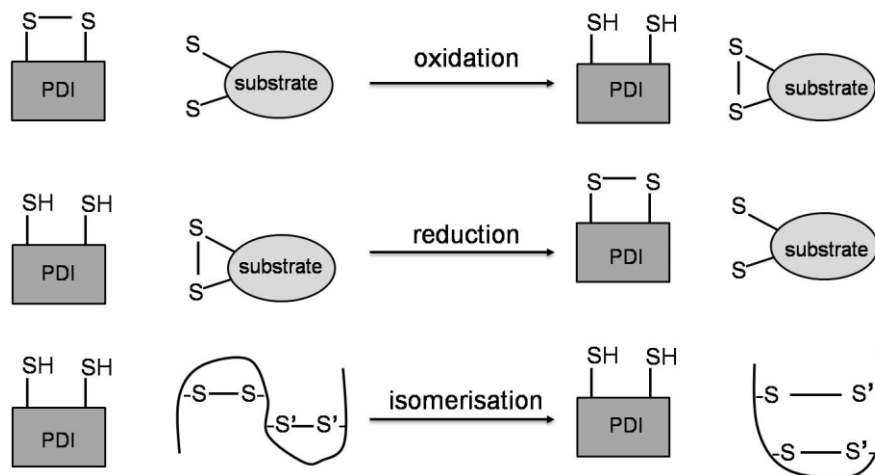


Figure 1.4. Thiol-disulphide exchange reaction. (A) Deprotonation of the free thiol results in generation of a thiolate anion (S^-) and this highly reactive moiety attacks a disulphide bond in the oxidized species. This results in the formation of a mixed-disulphide bond between a protein and redox molecule or the two proteins. Subsequently in the so called escape pathway, the remaining thiolate anion attacks the mixed-disulphide bond and resolves it. The “disulphide exchange reaction” results in the oxidation of the originally reduced protein, and the reduction of the initially oxidized redox species. (B) The redox reactions catalysed by the CXXC motif within the catalytical domain of the oxidoreductase PDI, can result in oxidation, reduction or isomerisation of disulphide bonds with substrate proteins.

stability and final conformation is critical (Sevier and Kaiser, 2002). Accordingly, incorrect cysteine pairing can disrupt native protein conformations. Briefly, oxidative folding involves disulphide bond formation between the thiol group of two cysteines in a protein. *In vitro* this reaction can occur spontaneously by the loss of an electron from each of two coupled cysteine thiols and their donation to an acceptor such as oxygen (Sevier and Kaiser, 2002). *In vivo*, disulphide bond formation occurs predominantly via “thiol-disulphide exchange reactions” i.e. exchange of an electron between a sulfhydryl-containing substrate such as glutathione or oxidoreductases and molecules with free thiols (Figure 1.4A).

1.2.2.1 The oxidative environment of the ER

The environment of the ER in which MHC class I molecules fold is relatively oxidising hence favouring disulphide bond formation. The oxidising conditions are maintained by the glutathione redox regulatory system (Schafer and Buettner, 2001). Glutathione (GSH) is a tripeptide of glutamic acid, cysteine and glycine. Glutathione is able to reduce disulphide bonds by serving as an electron donor whilst its oxidised form, glutathione disulphide (GSSG) catalyses disulphide bond formation. Within the ER lumen the GSH:GSSG ratio is 1:1 to 3:1 providing a more oxidising environment in comparison to the cytosol, where the GSH:GSSG ratio is 30:1 to 100:1 (Hwang et al., 1992) (Bass et al., 2004). In the highly oxidising environment of the ER, disulphide bond reduction and isomerisation are crucial in preventing protein substrates from getting trapped in off-pathway intermediates on the way to attaining their native conformation (Jansens et al., 2002). Disruption of the glutathione redox regulatory system

equilibrium can affect both ER and cytosolic proteins (Braakman et al., 1992) (Abate et al., 1990) (Toledano and Leonard, 1991) (Marchetti et al., 1997) (Blazquez et al., 1996).

However, using *S. cerevisiae* to genetically dissect oxidative protein folding, it was shown that glutathione is not required for oxidative protein folding in eukaryotes (Frand and Kaiser, 1998). Moreover, *in vitro* disulphide-bond formation conducted by the system of ER oxidoreductase can proceed despite the excess of reduced glutathione (Tu et al., 2000). This GSH-independent system is based on the ER oxidoreductin 1 (Ero1), a conserved protein that generates disulphides in a flavin (FAD)-dependent reaction that consumes molecular oxygen. The oxidised form of Ero1p, generated when two electrons are donated into FAD, can in the disulphide exchange reaction directly transfer the oxidizing equivalents to the oxidoreductase PDI which, as an oxidised form can further facilitate disulphide bond formation within substrate protein (Frand and Kaiser, 2000) (Pollard et al., 1998).

However, lowering the level of GSH in the cell results in an increase in disulphide bond formation indicating that glutathione plays a role in the reductive pathway. Furthermore, lowering the levels of GSH correlates with an accumulation in non-native disulphide bond formation and an increase in the time required for disulphide bond isomerisation indicating a role for glutathione in the latter redox process (Chakravarthi and Bulleid, 2004) (Molteni et al., 2004).

Glutathione can either be directly or indirectly involved in native disulphide bond formation (Bass et al., 2004) (Jessop and Bulleid, 2004). By analysing GSH

binding to monobromobimane (mBBr), which is a thiol-reactive compound widely used to detect various thiol-containing biomolecules (Cotgreave and Moldeus, 1986) (Kosower and Kosower, 1995a) (Newton and Fahey, 1995), indicates that glutathione can form direct mixed disulphides with ER proteins both during reduction or oxidation (Bass et al., 2004). Furthermore, GSH can reduce the PDI family member ERp57 (Jessop and Bulleid, 2004) indicating that GSH may be involved in maintaining oxidoreductases in their reduced state allowing them to facilitate the reduction and isomerisation of non-native disulphide bonds within ER substrate proteins (Fratelli et al., 2002).

1.2.2.2 Thioredoxin family

PDI

Oxidoreductases are a group of enzymes that catalyze disulphide bond exchange reactions i.e. oxidation (formation), reduction (breaking) and isomerisation (rearrangement) *in vivo*. One of the most abundant ER resident oxidoreductases are members of the PDI family belonging to the Thioredoxin (Trx) superfamily of protein-oxidoreductases (Freedman et al., 1994). They all contain the redox-active domains within the catalytic CXXC motifs in which two active cysteine residues directly take part in the redox modification of thiols located within the substrate protein. Mutation of cysteines within CXXC motifs can result in loss of function indicating their catalytic activity (Antoniou et al., 2002).

PDI is composed of four Trx-like domains (a, b, b', a') where the two N- and C-terminal domains, a and a' respectively, each contain a CXXC motif (Edman et

al., 1985). The b and b' domains lack any active motifs and seem to be important for substrate interaction and proposed peptide binding activities of PDI (Kemink et al., 1997) (Klappa et al., 1998a) (Pirneskoski et al., 2004). The crystal structure of PDI revealed that its domains are arranged in the shape of a twisted "U" with active sites localised at the two flexible arms (Li et al., 2006) (Tian et al., 2006) (Tian et al., 2008). Such molecular flexibility would ensure accommodation of structurally diverse substrates (ranging in size from insulin (5.7 kD) to procollagen (~100 kD)) (Wilson et al., 1998) and may also be needed during the isomerization reaction to ensure that the active sites can locate mispaired disulphide bonds in the substrate. The inside surface of the U is hydrophobic which is thought to aid in recognition of misfolded molecules (Tian et al., 2008).

Depending on the substrate and redox environment, PDI can catalyse reduction, formation and isomerisation of disulphide bonds (Figure 1.4B). When the cysteines within the active site of PDI are in a reduced state, PDI can catalyse reduction of the oxidised substrate. Firstly, deprotonation of the N-terminal cysteine within the CXXC motif leads to the generation of a highly reactive thiolate anion that attacks disulphide bonds (Figure 1.4A). This results in the formation of a transient PDI-substrate mixed disulphide species. In the second exchange reaction, called the escape pathway, the remaining thiolate anion within the CXXC motif attacks the mixed disulphide bond which subsequently leads to its reduction. This results in reduction of the originally oxidised substrate and oxidation of PDI. Conversely when the PDI active site is in the oxidised state, disulphides can be transferred from PDI to the substrate

and its active site becomes reduced. The PDI oxidised state of the active CXXC unit can be regenerated due to action of the ER oxidoreductin 1 (Ero1). Ero1 is a luminal glycoprotein essential for the introduction of oxidising equivalents into the ER since overexpression of ERO1 confers resistance to otherwise toxic levels of the reducing agent dithiothreitol (DTT) (Frand and Kaiser, 2000). Detection of mix-disulphide intermediates between Ero1 and PDI indicated the presence of direct thiol-disulphide exchange reactions occurring between Ero1 and PDI resulting in PDI oxidation and subsequent protein substrate oxidation (Pollard et al., 1998) (Frand and Kaiser, 1998). Mutational analysis of Ero1 revealed that two pairs of cysteine residues (CXXCXXC) are responsible for the oxidising activity of this protein (Frand and Kaiser, 2000).

PDI homologues

The last few years have brought the discovery of new PDI family members which now consist of around 19 proteins (Appenzeller-Herzog and Ellgaard, 2008). The most well studied PDI family members are: ERp57 (Oliver et al., 1997) (Oliver et al., 1999) (Antoniou et al., 2002) (Peaper et al., 2005) (Santos et al., 2007) (Zhang et al., 2009) (Dong et al., 2009), PDIp (Desilva et al., 1996) (Volkmer et al., 1997) (Klappa et al., 1998b) (Ruddock et al., 2000), ERp72 (Mazzarella et al., 1990) (Miyaiishi et al., 1998) (Forster et al., 2006) (Menon et al., 2007), P5 (Chaudhuri et al., 1992) (Hayano and Kikuchi, 1995a), PDIr (Hayano and Kikuchi, 1995b), ERp44 (Anelli et al., 2002) (Anelli et al., 2003) (Wang et al., 2008), ERdj5/JPDI, (Cunnea et al., 2003) (Ushioda et al., 2008) (Thomas and Spyrou, 2009), ERp29/28 (Demmer et al., 1997) (Mkrtchian et al., 1998) (Hermann et al., 2005) (Rainey-Barger et al., 2007) (Barak et al., 2009),

PDILT (van Lith et al., 2005) (van Lith et al., 2007) and smallest ERp18 (Alanen et al., 2003). The common feature of the PDI family members is the presence of a domain with a Thioredoxin-like fold. The number and organisation of these domains vary between protein family members as well as their client substrates and partner binding sites (Kozlov et al., 2010).

Interestingly, possessing catalytically activity does not determine PDI family affiliation since ERp27/29 lacks a CXXC motif (Ferrari et al., 1998) (Alanen et al., 2006). Moreover, protein disulphide isomerase-like protein of the testis (PDILT) contains an unusual, SXXC, thiol-reactive motif lacking an N-terminal cysteine. Although PDILT has been shown to form mixed disulphides with partners and substrates *in vivo* via the remaining C-terminal cysteine residue, it is not fully understood whether it is involved in oxidative protein folding (van Lith et al., 2005). It seems that rather the chaperone function of PDILT is more important in a spermatogenesis-specific chaperone system with the testis-specific calnexin homolog, calmegin (van Lith et al., 2007). Similarly, ERp44 contains a single non-canonical CXXS thiol-reactive motif. Despite it being found to interact with Ero1 its direct involvement in oxidative protein folding might be secondary. Instead, ERp44 influences the concentration of Ca^{2+} in the ER by regulating the activity of the Inositol 1,4,5-trisphosphate receptors (IP3Rs) calcium channels (Higo et al., 2005). Since several chaperones, such as calreticulin, rely upon Ca^{2+} binding to regulate protein-protein interactions, ERp44 seems to facilitate productive protein folding regardless of catalytical activity (Michalak et al., 2009).

ERp57

ERp57 (GRP58) is the closest homologue to PDI since it possesses similar domain organisation and shares 29% sequence identity and 56% similarity in primary structure with PDI (Koivunen et al., 1996). Likewise PDI, it is composed of two thiol-reactive domains a and a' each containing a canonical CXXC motif spaced by two, b and b' non-active domains. It also possesses thiol-dependent reductase activity and forms mixed disulphides with protein substrates. However, ERp57 can not substitute for PDI since ERp57 is thought to almost exclusively influence the redox state of glycoproteins since it was shown to bind to integral membrane protein glycophorin C (GlyC) with intact N-linked oligosaccharide side chains but not to its non-glycosylated mutant version (Koivunen et al., 1996) (Elliott et al., 1997) (Oliver et al., 1997) (Ellgaard and Frickel, 2003). Moreover, substrate recognition is more sophisticated in the case of ERp57. Cross-linking studies identified non-covalent interactions between ERp57 and either calnexin or calreticulin as these lectin proteins recruit ERp57 via their extended proline rich domain (P domain) (Russell et al., 2004) (Oliver et al., 1999). Inhibition of lectin-substrate interactions by castanospermine abolishes ERp57 recruitment resulting in altered oxidative folding of model viral glycoproteins (Molinari and Helenius, 1999) (Solda et al., 2006). Hence calnexin and calreticulin act as adaptor molecules that define the substrate specificity of ERp57. Furthermore, ERp57 preferences toward monoglucosylated glycoprotein substrates is significantly enhanced *in vitro* when CNX or CRT is present (Zapun et al., 1998). The most intensively studied example of ERp57 activity is its involvement in folding and maturation of MHC

class I molecules (Hughes and Cresswell, 1998) (Morrice and Powis, 1998) (Lindquist et al., 2001).

1.2.2.3 MHC class I Oxidative Folding

All MHC class I molecules possess four conserved cysteine residues at p101, p164, p203 and p259 which form two structurally important disulphide bonds within the $\alpha 2$ - and $\alpha 3$ -domain respectively (Ribaudo and Margulies, 1992) (Wang et al., 1994) (Figure 1.1). Appropriate disulphide bond formation is crucial, since mutagenesis of cysteines compromising either disulphide results in a decreased rate of maturation of the heavy chain, reduced cell surface expression and impaired peptide loading (Shiroishi et al., 1984) (Miyazaki et al., 1986) (Warburton et al., 1994). Hence, within the oxidizing environment of the ER the empty MHC class I molecule is prone to undergoing redox regulation by oxidoreductases (Gilbert, 1997) (High et al., 2000). Detection of transient disulphides between class I heavy chain and three oxidoreductases: PDI, ERp57, and ERp72 allowed to identify them as major facilitators of the MHC class I oxidative folding pathway (Antoniou et al., 2002) (Lindquist et al., 2001) (Park et al., 2006) (Chambers et al., 2008) (Santos et al., 2007). ERp57 is the master oxidoreductase during MHC class I assembly. It was detected in association with both $\beta 2m$ -free and $\beta 2m$ -associated heavy chains as well as being a component of the PLC (Antoniou et al., 2002) (Farmery et al., 2000) (Lindquist et al., 2001).

1.2.2.3.1 Redox regulation of early MHC class I folding stages

The formation of conserved disulphide bonds occurs at different stages of class I folding. The α 3-domain disulphide bond forms rapidly after synthesis of the heavy chain into the lumen of the ER (Tector et al., 1997) (Ribaudo and Margulies, 1992) whilst the α 2-domain disulphide, which “locks” the α -helix to the β -pleated sheet of the antigen binding groove, was proposed to remain in a reduced state until antigen acquisition (Antoniou et al., 2002). As I mentioned before, the early folding events of MHC class I assembly are facilitated by the CNX cycle in concert with ERp57. The activity of ERp57 activity toward monoglucosylated glycoprotein substrates is significantly enhanced *in vitro* when CNX or CRT are present (Zapun et al., 1998) whilst inhibition of glucose trimming (with castanospermine) abrogates formation of ERp57-containing mixed disulphides during the oxidative folding of model viral glycoproteins (Molinari and Helenius, 1999). Despite these findings, ERp57 does not appear to require interactions with CNX/CRT in order to promote efficient MHC class I folding (Zhang et al., 2009).

Depletion of ERp57 by RNA interference slows down the formation rate of a fully oxidised α 3-domain suggesting that ERp57 influences the formation of the C203-C259 disulphide bond within newly synthesised heavy chains (Zhang et al., 2006b). Recently, it was proposed that PDI also plays a role in the early folding of MHC class I since it was found to associate with the heavy chain and catalyse disulphide bond formation prior to β 2m association with heavy chain (Kang et al., 2009). PDI-depletion experiments resulted in enhanced heavy

chain-CN χ interactions and impaired heavy chain associations with β 2m (Kang et al., 2009).

1.2.2.3.2 Redox regulation within the PLC and during peptide loading

Whether the α 2-domain disulphide bond remains partially reduced or oxidised upon entry into PLC remains uncertain. It was shown that full oxidation of the heavy chain can occur prior to association with β 2m, although the presence of β 2m can promote stability and enhance the efficiency of disulphide bond formation (Tector et al., 1997).

Most studies so far have demonstrated that formation and maintenance of the α 2 disulphide bond is a structural requirement for β 2m association, recruitment of the heavy chain into the PLC and optimal peptide loading (Warburton et al., 1994) (Ribaldo and Margulies, 1992) (Wang et al., 1994) (Smith et al., 1995) (Tector et al., 1997). Although, several studies support the idea that MHC class I heavy chain- β 2m complexes enter the PLC as fully oxidised molecules, further assembly and peptide optimisation is assisted by oxidoreductases suggesting that final folding events are redox dependent. It was also proposed that the empty/peptide-receptive MHC class I is prone to attack by ERp57 and/or PDI (Antoniou et al., 2002). Consistent with this assumption, *in vitro* studies revealed the α 2-domain disulphide of the unassembled MHC class I heavy chain was found to be susceptible to reduction by ERp57 whilst peptide-loaded heavy chain was resistant (Antoniou et al., 2002).

Function of the ERp57-tapasin complex

Within the PLC, ERp57 associates with CRT via its b' domain and forms a stable mixed disulphide with tapasin that involves C57 of ERp57 and C95 of tapasin (Peaper et al., 2005) (Figure 1.5). It was estimated that up to 80% of the cellular pool of ERp57 forms a heterodimer with tapasin (Peaper et al., 2005). The very stable ERp57-tapasin conjugate is unique since disulphide intermediates between oxidoreductases and their substrate are normally transient. The escape pathway, which usually prevents accumulation of conjugates (Sevier and Kaiser, 2002), in the case of the ERp57-tapasin dimer, is prevented by a covalent interaction between these two molecules. Disruption of the ERp57-tapasin conjugate by mutation of C95 in tapasin prevented formation of the ERp57-tapasin heterodimer and resulted in partially reduced class I heavy chains (Dick et al., 2002). Hence, it was proposed that tapasin binding to ERp57 within the PLC prevents ERp57-mediated reduction of the $\alpha 2$ -domain disulphide of the heavy chain, therefore maintaining the peptide binding groove in a peptide-receptive state (Kienast et al., 2007). This hypothesis is in line with findings that cellular ERp57 is predominantly in a reduced state and hence its activity is limited to disulphide bond reduction or isomerisation (Antoniou and Powis, 2003) (Jessop et al., 2007). This raises the question concerning the role of ERp57 in oxidative folding of MHC class I heavy chains within the PLC. The importance of ERp57 for the PLC function was emphasized by Garbi et. al. who found that in B cells lacking expression of ERp57, the incorporation of MHC class I heavy chain into the PLC was impaired and more transient. Moreover, the maturation rate and trafficking through the Golgi was

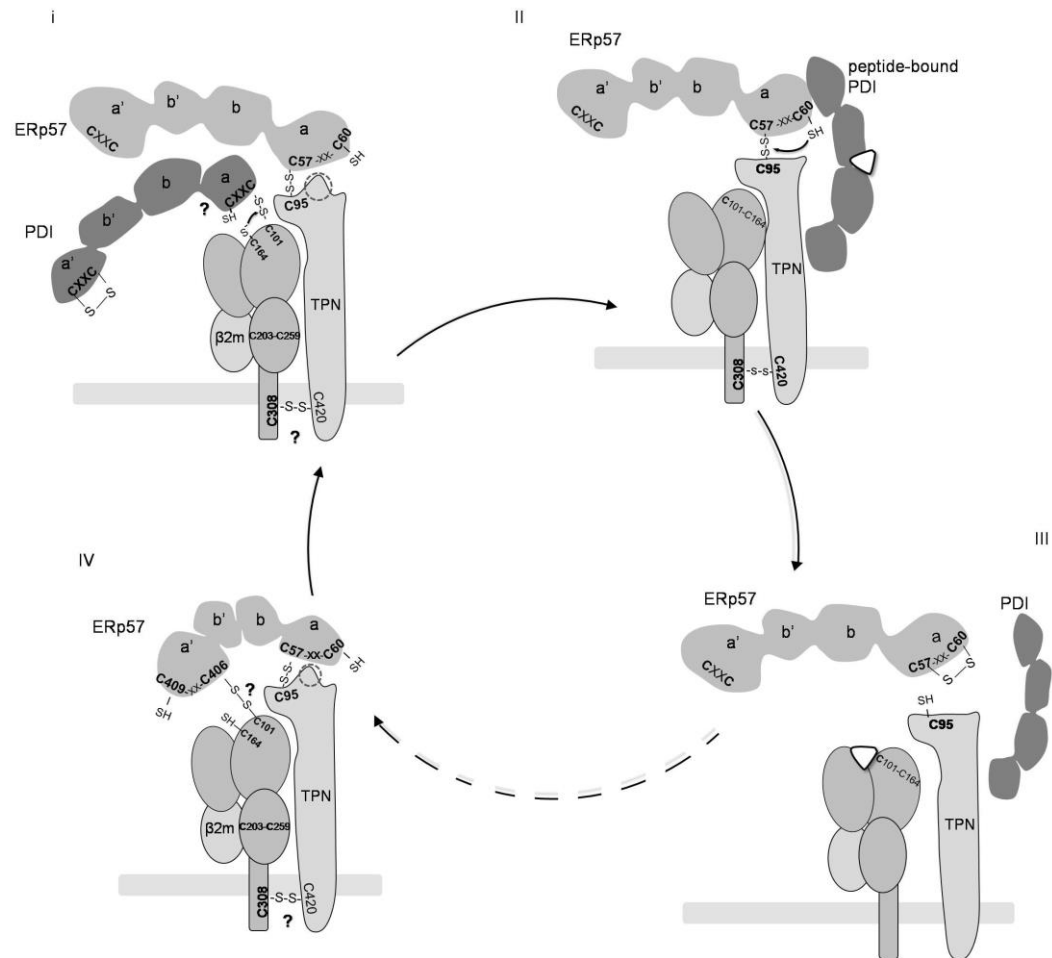


Figure 1.5. Redox regulated peptide loading. (I) Prior to peptide binding C95 of tapasin (TPN) is disulfide bonded to C57 from the a-domain CXXC motif of ERp57 to form a stable conjugate and C60-mediated escape pathway is prevented by the noncovalent interaction between ERp57 and TPN (dotted circle). Potentially, disulphide bonding between C420 of tapasin and C308 of heavy chain can also exist. PLC associated PDI could mediate oxidation of the C101-C164 heavy chain disulphide making the heavy chain vulnerable to peptide acquisition. (II) PDI transfer of peptide to the fully oxidised heavy chain and its binding to the PLC initiates conformational changes of TPN that lead to activation of the escape pathway mediated by C60 of ERp57. (III) This can lead to dissociation of the TPN-ERp57 conjugate and release of MHC class I loaded with optimal peptide. (IV) The C101-C164 disulphide bond of the heavy chain loaded with suboptimal peptide could retain susceptibility to reduction plausibly mediated by ERp57 (CRT and TAP were excluded from the figure for simplification).

accelerated, however cell surface expression and thermostability was diminished indicating suboptimal peptide loading (Garbi et al., 2006). Alternatively the observed maturation rate could be the result of the lack of CRT in the PLC in the absence of ERp57 since this phenotype is similar to the CRT knockout cells and the T134K class I mutant that exits the ER prematurely (Gao et al., 2002) (Lewis and Elliott, 1998). Remarkably, there was no alteration in redox state of the PLC-associated heavy chain. Hence it remained questionable whether ERp57 requires its enzymatic activity to function within the PLC. To address this issue, Zhang et al generated an ERp57 mutant with only C57 intact, which therefore lacked oxidoreductase activity but remained capable of associating with tapasin. In the presence of this ERp57-mutant, peptide acquisition by MHC class I heavy chains was as efficient as in the presence of wild-type ERp57, suggesting that ERp57 function in the PLC is rather structural and its catalytical activity is dispensable for promoting the loading of optimal peptides into MHC class I heavy chain (Zhang et al., 2009).

Tapasin itself possesses five conserved cysteine residues, two within the Ig-like region (C295 and C362) and three conserved cysteine residues that lie within N terminal of the Ig region (C7, C71 and C95). As mention before, C95 is engaged in a disulphide bond with ERp57. Interestingly, disruption of C7 and C71 that form an intrachain disulphide bond within tapasin, reduces the formation of the tapasin-ERp57 conjugate (Dick et al., 2002). The two cysteines at p295 and p362 form a structurally important disulphide bond within an Ig-like domain, that is important for tapasin interaction with both MHC class I heavy chain and TAP (Turnquist et al., 2004).

PDI in the PLC

In comparison with ERp57 whose function seems to be limited to disulphide bond reduction and/or isomerisation, PDI can reduce, oxidise and isomerise disulphide bonds (Noiva, 1999) (Lundstrom and Holmgren, 1993). Hence, its presence within the PLC complex which was demonstrated by co-immunoprecipitation and mass spectrometry, suggested that it might be responsible for MHC class I oxidation during peptide loading (Figure 1.5A). Indeed, its catalytical activity was found to be critical in stabilizing a peptide-receptive site by regulating the oxidation state of the $\alpha 2$ disulphide bond in the MHC class I peptide-binding groove (Park et al., 2006). Moreover, PDI was found to promote optimal peptide binding by increasing the speed and efficiency of antigen loading as demonstrated by the acquisition of EndoH resistance and thermostability respectively (Park et al., 2006). Remarkably, both the maturation rate and optimal peptide binding were restored in the presence of a PDI-mutant lacking the a' domain indicating that the chaperoning function of PDI was also required for efficient MHC class I assembly (Park et al., 2006).

However, the presence of PDI within the PLC has been questioned. Santos et al. reported that since the interaction of PDI with the PLC was not consistent in their experimental system, PDI might associate with the PLC more transiently with a relatively low affinity reaction, hence the association of PDI within the PLC requires further investigation (Santos et al., 2007).

PDI as a peptide-carrier

PDI has also been proposed to be a potential peptide-carrier that delivers TAP-translocated antigens directly to the MHC class I heavy chain (Figure 1.5).

Binding and release of peptide substrates was proposed to be regulated by the redox state of PDI in a manner similar to that observed during the unfolding of Cholera Toxin (Tsai et al., 2001). Cholera Toxin, produced by *Vibrio cholerae*, is an oligomeric protein composed of one catalytically active A subunit and five B subunits. In order to activate the toxin, its A subunit must be detached from the B subunits and be translocated from the ER to the cytosol of the invaded intestinal epithelial cells. Translocation of the A subunit requires its unfolding which is mediated by PDI as proposed by Tsai et al (Tsai et al., 2001). Notably, only in a reduced state can PDI bind toxin leading to the reduction of intrachain disulphide bonds and protein unfolding whilst PDI oxidation, leads to toxin release (Tsai et al., 2001). Based on this example it is plausible that PDI-mediated peptide delivery to the MHC class I heavy chain might also be dependent on the PDI redox state. However, such a scenario would require alterations in the redox state of PDI within and outside the PLC. Nevertheless, since there are also reports of a PDI-substrate binding cycle that is independent of PDI redox state (Lumb and Bulleid, 2002), the mechanism involved in the binding and release of PDI substrates requires further studies.

Taken together, the $\alpha 2$ -domain disulphide bond, due to its location in proximity to the peptide binding groove, can potentially influence peptide binding and conversely, peptide acquisition could “protect” (cover) the disulphide bond, thus making it less susceptible to reduction. Support for the former is suggested by the removal of C101 or C164 impairing optimal peptide loading and cell surface expression of HLA-A*02:01 (Warburton et al., 1994). On the other hand, blocking the delivery of peptides into the ER increases accessibility of the $\alpha 2$

domain disulphide to reduction (Park et al., 2006). Based on the existing evidence, the model of redox regulated peptide editing was proposed in which, prior to optimal peptide binding, the $\alpha 2$ domain disulphide is in dynamic equilibrium between an oxidised and reduced state. The activity of PDI results in oxidation of the C101-C164 bond and adoption of the peptide-laible conformation by the peptide binding groove. Subsequently, PDI can transfer peptide to the fully oxidised heavy chain. The binding of optimal peptide would bury the $\alpha 2$ domain disulphide bond (Bjorkman et al., 1987) making it resistant to reduction whilst sub-optimally loaded/empty heavy chain would be susceptible to reduction potentially mediated by ERp57. Dissociation of the reduced MHC class I heavy chain would allow for further rounds of PDI-mediated re-oxidation and peptide binding (Kim et al., 2009).

1.2.2.3.3 Redox-regulated MHC class I export

The main, but insufficient requirement for release of MHC class I from the PLC and its further trafficking to the cell surface is optimal peptide loading. It seems that the tapasin-ERp57 heterodimer is crucial in regulating MHC class I retention/egress from the ER. Both in the absence of tapasin or ERp57 acquisition of optimal peptide is impaired but MHC class I exit from the ER is not affected but even accelerated (Garbi et al., 2006) (Grande et al., 2000). Interestingly, the tapasin-mediated retention of MHC class I molecules seems to be specific to empty but not peptide loaded heavy chain. Hence, the tapasin-ERp57 disulphide conjugate is crucial in preventing egress of empty MHC class I molecules from the ER. Furthermore, Lee et al. show that disruption of the

ERp57-tapasin conjugate can trigger MHC class I disassembly which is conducted by PDI (Lee et al., 2009). Lee et al proposed a model whereby PDI acted as a peptide dependent molecular switch in which PDI that binds to peptide, interacts with tapasin-ERp57 inducing their detachment, whilst peptide-free PDI is unable to bind to the tapasin-ERp57 heterodimer. The interaction of peptide-bound PDI with tapasin-ERp57 may trigger structural changes within this complex that could restore reducing activity of C60 within ERp57 and activate the escape pathway. Interestingly, in PDI-overexpressing cells, MHC class I acquisition of optimal peptide was not altered as measured by the acquisition of thermostability, but exit of the heavy chain from the ER was significantly impaired probably due to an increase in the peptide-free PDI pool. Hence, MHC class I disassembly is not a default event but is a regulated process with optimal peptide binding itself not sufficient to trigger MHC class I egress from the ER.

1.3 Protein misfolding

Schubert et al estimated that around 30% of newly synthesized proteins misfold during early stages of their assembly (Schubert et al., 2000). Protein misfolding is hence relatively common and it is an intrinsic propensity of proteins that occurs constantly. Moreover, the loss of native conformation may also occur due to protein-modifying conditions that, can have environmental sources such as exposure to increased temperature, high or low pH or oxidative agents (Azuaga et al., 2002) (Iametti et al., 1996) (Giri et al., 2007) (Vetri et al., 2007) (Fan et al., 2008) (Fan et al., 2007) (Carneiro et al., 2001) (Rakhit et al., 2002)

(Stewart et al., 2005b) (Stewart et al., 2007). The failure of proteins to fold appropriately is associated with a large number of cellular disorders that may give rise to diseases. Within healthy cells there are several protein quality control systems such as the CNX/CRT cycle or BiP, which facilitate proper folding. However, when protein quality control machinery is congested it may lead to either degradation of defective molecules or their accumulation and subsequent formation of toxic aggregates.

1.3.1 Endoplasmic Reticulum associated degradation (ERAD)

Degradation of misfolded proteins within the ER is facilitated by a process known as Endoplasmic Reticulum associated degradation (ERAD) that targets defective molecules for retrotranslocation from the ER followed by proteolytic degradation in the cytosol (Kostova and Wolf, 2003) (Nakatsukasa and Brodsky, 2008). Essentially, the role of ERAD is to remove terminally misfolded proteins in order to maintain homeostasis within the ER.

ERAD substrate recognition

Firstly, misfolded protein must be recognised and distinguished from correctly folded molecules or folding intermediates. Indeed there is little known about the mechanisms that target proteins to be retrotranslocated from the ER. It seems that there are several levels/check points for misfolded protein recognition. The main mechanism of highlighting proteins destined for degradation involves monitoring post-translational modifications such as N-glycosylation in case of glycoproteins. The protein is recognised as a substrate for degradation when a

single mannose residue is removed from the core oligosaccharide. First mannose trimming reduces a $\text{Man}_9\text{GlcNAc}_2$ oligosaccharide to $\text{Man}_8\text{GlcNAc}_2$, and is conducted by ER-mannosidase I enzyme, as its deletion or mutations leads to impaired glycoprotein degradation (Su et al., 1993) (Knop et al., 1996) (Vallee et al., 2000) whilst overexpression enhances turnover of glycoproteins (Helenius and Aebi, 2004) (Wu et al., 2003). At the same time mannose trimmed proteins are weak substrates for reglucosylation, preventing their re-entry into the calnexin/calreticulin cycle for further rounds of folding. The presence of $\text{Man}_8\text{GlcNAc}_2$ on terminally misfolded glycoproteins is a binding signal for ER-degradation enhancing α -mannosidase-like protein (EDEM1) (Helenius and Aebi, 2004). EDEM1 accelerates degradation of terminally misfolded glycoproteins (Hosokawa et al., 2001) (Hosokawa et al., 2006) by extracting misfolded proteins retained in the calnexin cycle (Molinari et al., 2003). Hosokawa et al. observed that overexpression of EDEM1 accelerated degradation of the alpha1-antitrypsin variant null (Hong Kong) (NHK) protein that would otherwise form hard to dispose off, aberrant disulphide linked dimers (Hosokawa et al., 2006). Hence EDEM1 prevents aggregation of misfolded proteins (Hosokawa et al., 2006) which due to exposure of hydrophobic regions are prone to aggregation thus impairing retrotranslocation (Fagioli et al., 2001). Since, EDEM1 does not seem to possess oxidoreductase activity itself, it has been suggested that it recruits other chaperones with the ability to reduce disulphide bonds. In support of this, the ER resident oxidoreductase ERdj5 was found to physically bind to EDEM1 and reduce disulphide bonds of misfolded proteins (Ushioda et al., 2008).

However, since mannose trimming is also a signal for efficient release of fully folded proteins from the ER, it is obvious that additional regulation for recognition of defective molecules exists. As ER chaperones assist in protein assembly they are in a good position to distinguish folding intermediates from terminally misfolded proteins and initiate targeting to degradation. Depending on whether misfolding affects luminal or cytoplasmic domains, both ER and/or cytosolic chaperones can be involved in degradation by ERAD respectively (Vashist and Ng, 2004).

Keeping proteins in a retrograde competent state

Among the ER luminal chaperones, BiP was found to associate preferentially to exposed regions of ERAD substrates (Schmitz et al., 1995) and seems to be essential for stabilising and maintaining the solubility of defective proteins (Brodsky et al., 1999). Nishikawa and colleagues have found that elevated temperature leads to aggregation of ERAD substrates when BiP was mutated (Nishikawa et al., 2001) whereas BiP overexpression enhances protein solubility (Kabani et al., 2003).

For efficient retrotranslocation, it was proposed that unfolding of the ERAD substrate, including intra- and interchain disulphide bond reduction was a necessary prerequisite (Tortorella et al., 1998) (Fagioli et al., 2001). However, whether the reduction of disulphide bonds in ERAD substrates occurs is debatable. MHC class I degradation mediated by the Human Cytomegalovirus (HCMV) encoded gene products US2/US11 (Wiertz et al., 1996a) was found to be impaired following the use of agents that altered the redox potential and/or free thiol status such as diamide or N-methylmaleimide (NEM) respectively

(Tortorella et al., 1998). Accordingly, the reducing agent DTT did not alter the rate of class I heavy chain degradation (Wiertz et al., 1996b). Intriguingly, the oxidising agent diamide, that can mediate disulphide bond formation was observed to inhibit translocation of another transmembrane protein, the TCR α chain, whether expressed as a full-length molecule or as a mutant lacking cysteine residues (Tortorella et al., 1998). This latter observation suggests that the redox potential may influence the retrotranslocation of ERAD substrates via other mechanisms irrespective of direct disulphide bond reduction. Nevertheless, EDEM1 was found to directly associate with the ER oxidoreductase ERp57 (Ushioda et al., 2008) whereas PDI were found to be a crucial component of the protein degradation machinery (Molinari et al., 2002). In the case of PDI, it seems that both enzymatic and chaperone like properties are involved in efficient retrotranslocation of ERAD substrates (Molinari et al., 2002). Using a yeast, cell-free system, Gillece and colleagues have shown that mutations in the catalytic sites of PDI impaired the export and degradation of disulphide-rich ERAD substrates, but not of thiol-free protein (Gillece et al., 1999) indicating that PDI is responsible for the reduction of disulphide bonds prior to retrotranslocation (Gillece et al., 1999). However, deletion of the PDI domain region responsible for binding peptide, resulted in impaired retrograde transport of a substrate lacking cysteines implicating its chaperone-like features in ERAD (Gillece et al., 1999).

Finally, considering the aperture and dimensions of the translocation channel (Johnson and van Waes, 1999), these can probably accommodate both glycosylated substrates and structures induced by disulphide bonding. Thus it is

likely that protein reduction is not an obligate step for retrotranslocation into the cytosol.

Export from the ER and degradation

Proteins recognised as substrates for ERAD are subsequently translocated from the ER and ubiquitinated prior to degradation. Whether proteins prior to retrotranslocation need to be fully unfolded is debatable. The export channel has been proposed to be formed by the Sec61 translocation channel i.e. the same pore complex through which proteins are translated during biosynthesis. Sec61 was identified as a main retrotranslocon on the basis of studies by Wiertz and colleagues. They demonstrated that HCMV gene products US2/US11 conduct degradation of MHC proteins through Sec61 (Wiertz et al., 1996b). Further experiments involving blocking (Schmitz et al., 2000) or mutation of Sec61 (Pilon et al., 1997) (Plempner et al., 1997) supported its role in mediating export of defective molecules. However, the export process depended on the topology of the defective protein. Although degradation of soluble ERAD substrates seems to rely strongly on the Sec61 channel, the export of transmembrane proteins seems to be Sec61-independent (Walter et al., 2001). Retrotranslocation of the tail anchored membrane protein Ubc6p was not affected by mutations within Sec61 (Walter et al., 2001). Moreover, it was independent from other ERAD components, particularly Der1p (Degradation in the ER protein 1) (in mammals Derlin-1) (Walter et al., 2001). Derlin-1, is an alternative candidate proposed to mediate ERAD substrate export from the ER as it is essential for the degradation of class I MHC molecules triggered by the US11 viral protein (Lilley and Ploegh, 2004).

In order to physically retrotranslocate ERAD substrates from the ER, the yeast homolog of Derlin-1, Derp1 forms a complex with Cdc48/Ufd1p/Np14p components of the ubiquitination and degradation machinery which altogether coordinate substrate recruitment for polyubiquitination (Ye et al., 2001b) (Jarosch et al., 2002). Ubiquitination is a post-translational modification carried out by a set of three enzymes, E1, E2 and E3. Briefly, ubiquitin is initially activated by ubiquitin-activating enzyme E1 by ubiquitin formation of a mixed anhydride with AMP (ubiquitin adenylate). Ubiquitin transfer to the E1 active site cysteine residue is accompanied by release of AMP. The ubiquitin molecule is then passed on to the active site cysteine of a ubiquitin-conjugating enzyme E2 via a trans(thio)esterification reaction, before reaching the final enzyme, E3. The E3 enzyme is a protein ligase, which recognises and binds to the target substrate protein followed by tagging it with the activated ubiquitin (Bays et al., 2001) (Sato et al., 2009). The process is then repeated and the presence of short (3-6) ubiquitin chains on substrate proteins is a signal for its delivery to the 26S proteasome (Shamu et al., 2001). There are several molecules identified to bind ubiquitin conjugates and potentially escort substrate proteins to the 26S proteasome. Two such ubiquitin chain receptors, Rad23p and Dsk2p seem to be crucial as their deletion significantly impairs ERAD (Medicherla et al., 2004) (Richly et al., 2005) (Verma et al., 2004). Subsequently, substrates undergo deubiquitination followed by proteasomal degradation of the misfolded molecules. Upon reaching the cytosol, misfolded proteins can be polyubiquitinated by the E3 ubiquitin ligase Hrd1p (Bordallo et al., 1998) (Hampton, 2002) and the E2 ubiquitin conjugating enzyme Ubc1p/ Ubc6p /Ubc7p. Alternatively the Doa10p (E3) ligase can ubiquitinate a misfolded

proteins. Doa10p acts on a much broader range of substrates and works in concert with Ubc6p E2 conjugating enzyme (Hampton, 2002) (Kostova et al., 2007).

1.3.2 ER stress and Unfolded Protein Response

Cells are exposed to a variety of environmental stressors throughout their life span, such as free radicals, radiation, hypoxia, pathogens, toxins etc. Protein misfolding within the ER can be considered an endogenous source of stress, which affects the ER compartment and is known as ER stress. Maintaining homeostasis within the ER between protein synthesis and degradation is crucial for cell viability. When the ERAD system is overwhelmed by accumulation of misfolded protein and fails to remove defective molecules, then ER stress ensues. To counterbalance ER stress, the cell activates multiple signalling pathways termed the UPR (Mori, 2003) (Patil and Walter, 2001). Essentially, the role of the UPR is to restore the favourable folding conditions within the ER via a series of different events which include reducing translocation of new proteins, increasing retrotranslocation and degradation of ER-localized proteins as well as enhancing the protein-folding capacity of the ER. However, when severity and/or duration of the stress accelerates, the outcome of the UPR may be apoptosis.

1.3.2.1 UPR

The UPR of higher eukaryotic cells possesses a three-pronged signal transduction axes mediated by three distinct ER-localised transmembrane kinases (Mori, 2003) (Patil and Walter, 2001): IRE1, pancreatic ER kinase

(PERK) and activating transcription factor 6 (ATF6) (Cox et al., 1993) (Mori et al., 1993). These three stress sensors remain in an inactive state due to the association of their ER luminal domains with BiP (Bertolotti et al., 2000) (Shen et al., 2002a). During ER stress BiP is sequestered away to unfolded proteins thus activating these effector molecules leading to their trans-autophosphorylation and oligomerization. These effector molecules subsequently initiate a transcriptional programme that attenuates ER stress by increasing expression of chaperones that enhance protein folding and inhibit protein translation to reduce the ER folding load while the degradation of unfolded proteins is increased (Lee et al., 2003) (Shaffer et al., 2004) (Sriburi et al., 2004)(Haze et al., 1999) (Zinszner et al., 1998) (Okada et al., 2002) (Harding et al., 2003) (Marciniak et al., 2004) (Silva et al., 2005) (Cao et al., 2012) (Hu et al., 2012) (Lee et al., 2002) (Yamamoto et al., 2007) (Adachi et al., 2008).

IRE1

The Ire1 pathway was firstly described in yeast. The genetic screen of the budding yeast *Saccharomyces cerevisiae* led to the identification of IRE1 as an essential conductor under conditions that trigger the UPR (Cox et al., 1993) (Mori et al., 1993). In comparison with higher eukaryotic cells where the UPR possesses a three-pronged signal transduction axes, the Ire1 pathway is the only know UPR activation pathway in yeast.

Human IRE1 cDNA was firstly isolated by Tirasophon and colleagues using primers based on a yeast conserved Ire1 domain. Mammalian IRE1 is homologous to the yeast Ire1p UPR (Cox et al., 1993) (Mori et al., 1993) but

displays several functional and structural differences. Although the ER luminal domain of mammalian IRE1 has extensively diverged from the yeast Ire1p, their C-terminal domains are 34% identical at the amino acid level and display both kinase and ribonuclease activity (Tortorella et al., 1998). Mammalian IRE1 exist in two isoforms: IRE1 α , which is expressed in all cell types, and IRE1 β whose expression is mainly limited to gut epithelial cells (Wang et al., 1998) (Bertolotti et al., 2001). The overexpression of both IRE1 α and IRE1 β activates expression of BiP whilst overexpression of IRE β additionally increases transcription of another UPR target gene, CHOP (Tortorella et al., 1998) (Wang et al., 1998) (Halleck et al., 1997) (Price et al., 1992) (Nishitoh, 2012). Thus, analogous to ER stress in yeast, IRE1 is a proximal sensor of the UPR. On the other hand, in murine IRE1 α/β -deficient cells transcription of BiP, under ER stress conditions, is not abrogated indicating IRE1 is not the sole pathway inducing UPR in mammals (Lee et al., 2002) (Urano et al., 2000a).

The downstream target of IRE1 endoribonuclease activity is the mRNA encoding the transcription factor X-Box binding protein-1 (XBP1) (Fig. 1.6B). (Yoshida et al., 2001). Activated IRE1 proceeds to remove a 26-nucleotide long intron from the XBP1 mRNA. This subsequently leads to a shift in the reading frame that generates a new C-terminal end containing a potent transactivation domain (Yoshida et al., 2001) (Lee et al., 2002) (Calfon et al., 2002).

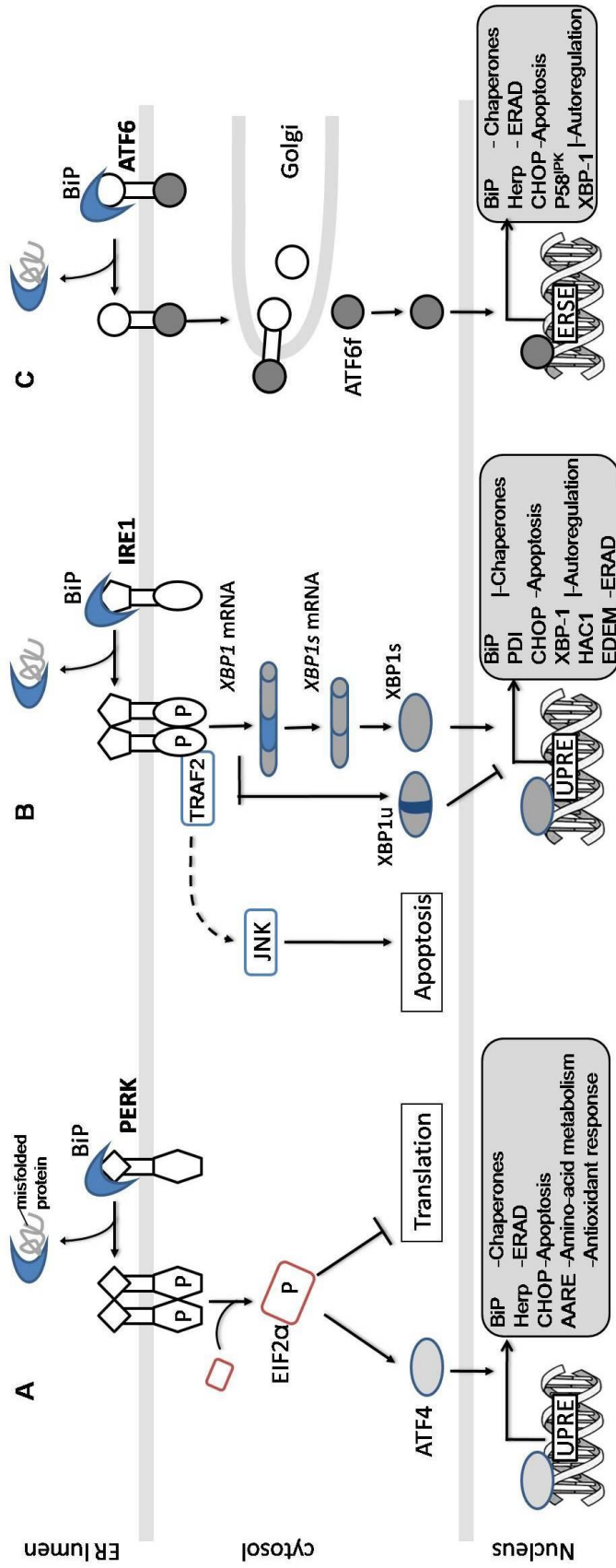


Figure 1.6. UPR signalling pathways. (A) During ER stress BiP dissociates from of PERK that phosphorylates (P) EIF2 α leading to protein translational arrest and activation of the ATF4. ATF4 translocates to the nucleus where it binds to UPR elements (UPRE) and activates UPR target genes including proapoptotic molecules. (B) BiP dissociates from IRE1 leads to oligomerisation and autophosphorylation of IRE1 which then excises the intronic sequence from XBP1 mRNA, generating spliced XBP1s mRNA that encodes a potent transcriptional activator (XBP1s). Unspliced XBP1 (XBP1u) protein can impair XBP1s transcriptional function. Phosphorylated IRE1 in concert with TRAF2 can activate JNK kinase that may promote apoptosis. (C) BiP dissociates from ATF6 allowing transit to the Golgi where it is cleaved leading to the release of ATF6f fragment (ATF6f) transcription factor. Within the nucleus ATF6f binds to ER-stress response elements (ERSE) inducing transcription of UPR target genes such as BiP, CHOP and XBP1.

The translated spliced version of XBP1 (XBP1s), is then translocated into the nucleus, where as a homodimer or heterodimer can associate with AFT6 α or NF-Y and binds to the promoter elements of many UPR-related genes (Lee et al., 2002) (Shaffer et al., 2004). Downstream targets of XBP1s include components of; (i) the ERAD system; EDEM1, Derlin1 and Derlin2, OS9, HERP; ER-localized chaperones such as DnaJ co-chaperones p58^{IPK}, ERdj4, human ER-associated DNAJ (HEDJ), protein disulphide isomerase P5 (PDI-P5) and Microvascular Endothelial Differentiation Gene 1 (MDG1), (ii) components of the secretory pathway, SEC23B, SEC24C, SEC61A, SEC61G, SRP54 and the translocating chain-associating membrane protein (TRAMP) and other genes related to cell viability such as Mist1 (Lee et al., 2003) (Shaffer et al., 2004) (Sriburi et al., 2004).

The mechanism regulating XBP1s activity is poorly defined. XBP1s was proposed to be negatively regulated by the unspliced version of XBP1 (XBP1u). The XBP1u protein is a short-lived protein (Tirosh et al., 2006) that in comparison with XBP1s, lacks the transactivation domain making it unable to initiate gene transcription. Yoshida and colleagues demonstrated that XBP1u can associate with XBP1s sequestering it away from the nucleus (Yoshida et al., 2006). Recent studies demonstrate that XBP1s undergoes posttranslational modifications such as acetylation, deacetylation or (Wang et al., 2011) sumoylation (Chen and Qi, 2010). The latter is the covalent attachment of small ubiquitin-like modifier (SUMO) protein whose ablation was found to significantly enhance the transcriptional activity of XBP1s (Chen and Qi, 2010). Despite

these promising findings, it is not known whether XBP1s activity is influenced by posttranslational modifications during ER stress.

The kinase activity of the IRE1 has yet to be fully characterised, however, it seems to be implicated in cell death in response to ER stress. Phosphorylated IRE1 was found to bind to the tumour necrosis factor (TNF)-receptor-associated factor 2 (TRAF2), that in turn activates the apoptosis signal-regulating kinase 1 (ASK1) pathway (Nishitoh et al., 2002). The IRE1-TRAF2-ASK1 complex subsequently promotes phosphorylation of JUN N—terminal kinase (JNK), which is impaired during ER stress in IRE1 α -deficient fibroblasts (Urano et al., 2000b). In turn, active JNK initiates pro-apoptotic mitochondrial-dependent caspase activation (Leppa and Bohmann, 1999).

PERK

The second UPR-controlling branch is mediated by the stress sensor PERK kinase, which shares several similarities with IRE1. In PERK deficient cells, there was impaired induction of numerous mRNAs that are responsible for the normal UPR indicating that PERK is an essential UPR component (Harding et al., 2003). PERK is a transmembrane molecule with an ER luminal domain tethered to BiP in resting cells. Under ER stress conditions BiP is thought to be recruited by misfolding molecules (Bertolotti et al., 2000), therefore initiating trans-phosphorylation of PERK which subsequently promotes PERK dimerisation. Activated PERK leads to phosphorylation and subsequent inactivation of eukaryotic translation-initiation factor 2 α (eIF2 α) causing translational arrest of most mRNA species (Harding et al., 1999) (Harding et al., 2000). Inhibition of protein synthesis is thought to attenuate ER stress.

Moreover, high levels of phosphorylated EIF2 α were shown to correlate with activation of nuclear factor (NF- κ B) (Jiang et al., 2003) (Deng et al., 2004). The mechanism of this activation is controversial as EIF2 α was found to mediate physical disruption of NF- κ B suppressor (I κ B) as well as I κ B synthesis inhibition respectively (Jiang et al., 2003) (Deng et al., 2004).

Additionally, EIF2 α phosphorylation increases the translation of ATF4 mRNA, by bypassing the two inhibitory upstream open reading frames (ORFs) localised within their 5' untranslated region (UTR) that normally prevents translation in resting cells (Lu et al., 2004) (Vattem and Wek, 2004). ATF4 encodes a bZIP transcription factor, which increases the transcription of genes involved in autophagy, apoptosis, amino acid metabolism and antioxidant responses (Zinszner et al., 1998) (Okada et al., 2002) (Harding et al., 2003) (Marciniak et al., 2004) (Silva et al., 2005) (Cao et al., 2012) (Hu et al., 2012). One of the main ATF4 target genes is the transcription factor CHOP (C/EBP homologous protein) that in turn can upregulate the expression of ER oxidase-1 (ERO1) (Marciniak et al., 2004). The main function of ERO1 is to maintain PDI in an oxidised state (Frand and Kaiser, 1998) (Pollard et al., 1998) thus allowing for disulphide bond formation during protein folding (Li et al., 2009). Additionally, ER stressed cells deficient in CHOP were found to be protected from the lethal consequences of ER stress (Marciniak et al., 2004). It was proposed that CHOP-mediated activation of GADD34, which in turn dephosphorylates EIF2 α , prevented translational attenuation and encouraged the recovery of ER load in stressed cells (Marciniak et al., 2004). Hence, the absence of CHOP protects

cells from ER stress by reducing ER client protein load and influencing redox conditions within the ER (Marciniak et al., 2004).

Regardless of activation by EIF2 α , ATF4 was found to be a critical regulator of osteoblast differentiation and function since ATF4^{-/-} mice showed delayed bone formation during embryonic development and low bone mass throughout postnatal life (Yang et al., 2004). This ATF4 activity could be of special importance regarding development of AS in which HLA-B27 misfolding and activation of the UPR were proposed as a disease mechanism (Turner et al., 2005).

PERK can also lead to activation of UPR target genes through ATF4-independent pathways (Blais et al., 2004) (Zhou et al., 2011). Cullinan et al. identified a second direct PERK substrate, a nuclear factor (erythroid-derived 2)-like 2 (Nrf2), that regulates oxidative cell responses (Cullinan et al., 2003) (He et al., 2001) (Cullinan and Diehl, 2004). Following exposure of cells to ER stress, PERK phosphorylates Nrf2, resulting in its dissociation from the cytoskeletal anchor Keap1. Activated Nrf2 is imported to the nucleus where it associates with other bZIP proteins: ATF4, c-Jun, Jun-B and Jun-D (Nguyen et al., 2003) upregulating expression of genes related to the oxidative stress response (He et al., 2001) (DeNicola et al., 2011). Hence, the PERK branch integrates distinct stress responses and indicates a link between protein misfolding and an imbalance in the redox state within the cell.

ATF6

ATF6 is a transmembrane transcription factor existing as an α and β isoform, which contains a bZIP motif within its cytoplasmic domain (Haze et al., 1999).

Similarly to IRE1 and PERK, the ATF6 luminal domain binds BiP in the absence of ER stress. Release of ATF6 from the ER was proposed to be regulated predominately by BiP binding (Shen et al., 2002a). Shen et al. immunoprecipitated ATF6-BiP complexes which disassemble as a result of ER stress. Removal of the luminal BiP binding sites or Golgi localization signals within ATF6 disrupted proper transport to the Golgi apparatus indicating that BiP retains ATF6 in the ER by inhibiting ATF6 Golgi localization signals (Shen et al., 2002a) (Sommer and Jarosch, 2002). However, a recent study postulated that, unlike in the case of IRE1 and PERK, disassociation of ATF6-BiP complexes are not a result of competitive binding of misfolded proteins to BiP but rather it is an active process (Shen et al., 2005). Hong et al. showed that the glucosylation status of ATF6 influences its release from the ER as an ATF6 mutation within the N-glycosylation site resulted in elevated levels of ATF6f and its constitutive transactivation. In resting cells ATF6 is fully glucosylated which is thought to promote calreticulin binding and ATF6 ER retention. Under ER stress conditions, underglycosylated ATF6 has been proposed not to associate with CRT, which is thought to promote transport of ATF6 to the Golgi apparatus (Hong et al., 2004). Hence, ATF6 may also serve as a sensor for ER homeostasis.

Another factor regulating ATF6 transport to the Golgi is related to its tendency to form disulphide bonded homodimers and oligomers via conserved cysteine residues within the luminal domain (Nadanaka et al., 2007). Reduction of intermolecular disulphides, although not sufficient, are required for ATF6

transition as the ATF6 monomer is the only form detected within the Golgi and is a better substrate for S1P (Nadanaka et al., 2007).

ATF6 translocated to the Golgi compartment (Schindler and Schekman, 2009) is cleaved by serine site-1 protease (S1P) and metalloprotease site-2 protease (S2P) leading to the release of the cytosolic bZIP domain of ATF6 (Chen et al., 2002) (Ye et al., 2000). The 50kD cytoplasmic fragment of ATF6 (ATF6f) translocates to the nucleus where it activates the transcription of target genes including ERAD components and XBP1 (Haze et al., 1999) (Lee et al., 2002) (Yamamoto et al., 2007) (Adachi et al., 2008). ATF6f binds to the ATF/cAMP response element (CRE) (Wang et al., 2000) and to ER stress response elements (ERSE-I and -II) (Kokame et al., 2001) (Yoshida et al., 1998) located in the promoters of target genes. Further studies by Li et al. revealed that optimal ATF6f stimulation requires at least 2 copies of ERSE and a nuclear factor Y (NF-Y). In thapsigargin-stressed cells, ATF6 was found to interact with another transcription factor YY1, which further enhanced ATF6 activity (Li et al., 2000). Moreover, ATF6 and XBP1s can share target DNA sequences and can be co-immunoprecipitated (Yamamoto et al., 2007), therefore explaining the partial overlap in UPR target genes between these two transcription factors (Wang et al., 2000).

1.3.2.1.1 Other roles of the UPR

Except for restoring the homeostatic control of protein folding, the UPR has been implicated in different physiological processes such as development and differentiation of distinct cell types and tissues and coordinates metabolic and immune responses (Reimold et al., 2001) (Martinon et al., 2010) (Richardson et

al., 2010) (Winnay et al., 2010) (Park et al., 2010). Particularly, the IRE1 branch of the UPR was seen to be important in development and maintenance of specialised secretory cells such as plasma cells (Reimold et al., 2001), exocrine acinar cells secreting digestive enzymes (Kaser et al., 2008), hepatic lipogenesis (Lee et al., 2008) and *in vitro* adipogenesis (Sha et al., 2009).

Terminal differentiation of B lymphocytes into plasma cells is a well studied physiological output of the UPR (Reimold et al., 2001). The population of plasma cells arise from the immature B cells that leave the bone marrow and are subjected to CD4⁺ T cell-mediated stimulation within secondary lymphoid organs. XBP-1 was found to be crucial in plasma cell differentiation i.e. synthesis of a large amount of immunoglobulins (Igs) accompanied by expansion of the ER. Mouse lymphoid chimeras deficient in XBP-1 showed impaired Ig secretion, impaired expression of CD138, and enhanced lethality due to infection by the B-cell-dependent polyoma virus (Reimold et al., 2001). Moreover, stimulation of plasma cell development was found to be accompanied by upregulation of XBP-1, induction of Xbp1 mRNA splicing and upregulation of UPR target genes such as BiP and GRP94 (Iwakoshi et al., 2003) (Gass et al., 2002). Accordingly, B cells deficient in IRE1 α displayed impaired antibody production although, they were also not able to progress from the earliest stage of B cells development (pro-B-cells) (Zhang et al., 2005) suggesting that the IRE1 branch of the UPR influences B cell development at different stages. Moreover, LPS-mediated B cell stimulation also results in cleavage of ATF6 (Gass et al., 2002) whilst expression of a dominant-negative ATF6 α mutant in differentiating B cells impaired secretion of the IgM (Gunn et

al., 2004). On the contrary, the PERK axis of the UPR does not seem to contribute to plasma cell development since PERK-deficient B cells differentiate into plasma cells (Gass et al., 2008) and stimulation of B cells does not lead to the activation of PERK downstream targets such as eIF-2 α (Ma et al., 2010). Taken together, these results demonstrate that a physiological UPR is required in terminal differentiation signals that allow B cells to produce vast quantities of antibodies and allow expansion of the secretory pathway, suggesting overlap between cellular differentiation and UPR pathways.

Recent studies have also reported the role of IRE1 in survival and development of Dendritic Cells (DC) (Iwakoshi et al., 2007), Paneth cell (Kaser et al., 2008) and macrophage proinflammatory responses (Martinon and Glimcher, 2011) suggesting the role of the UPR in regulation of innate immunity. Chimeric mice lacking XBP-1 have drastically low numbers of both conventional and plasmacytoid DCs and these display reduced survival both at baseline and in response to Toll-like receptor (TLR) stimuli. Accordingly, retroviral transduction of XBP-1 in bone marrow cells rescued and enhanced DC development (Iwakoshi et al., 2007). Recently, Martinon and colleagues reported that XBP-1 was required for optimal and sustained production of proinflammatory cytokines in macrophages. They found that regardless of ER stress, stimulation via TLR (2 and 4) upregulates the IRE1 branch of the UPR. XBP-1s was demonstrated to be responsible for cytokine production and thought to accelerate transcription of pro-inflammatory cytokines (such as IL-6, TNF, IFN- β) in macrophages (Martinon et al., 2010). The role of XBP-1 in proinflammatory responses was subsequently supported by studies where mice deficient in XBP-1 were unable

to efficiently eliminate bacterial pathogens (Martinon et al., 2010). Similarly, XBP-1 was found to be essential for Intestinal epithelial cell (IECs) function since XBP-1 deletion results in spontaneous enteritis and failure to generate antimicrobial activity i.e. diminished antimicrobial peptide secretion by Paneth cells and impaired responses to inflammatory signals in the local milieu (Kaser et al., 2008). Hence, the above observations support cross-talk between the UPR and innate immunity signalling.

1.3.3 Protein misfolding and disease

Diseases triggered by misfolded molecules can have several aspects. Some diseases have been categorised as protein misfolding/conformational disorders. Such diseases can trigger either a “gain of function” as a result of inappropriate folding or “loss of function” due to a specific mutation phenotype. Particularly, impaired disposal of defective molecules can lead to their accumulation into toxic aggregates. Protein aggregation can account for the development of disorders such as neurodegenerative disorders and amyloidosis whilst other disorders can be attributed to the loss of function of proteins, such as cystic fibrosis. The central aspect of these diseases is the presence of non-native protein structures whereas affected organs and tissues as well as symptoms may vary. Alternatively, defective molecules can compete with their functional counterparts (Goldberg, 2003) (Romisch, 2004).

Protein misfolding has been implicated in the pathogenesis of several human diseases such as Marfan syndrome (Whiteman et al., 2006), Alzheimer's disease, Parkinson's disease, α 1-antitrypsin deficiency, phenylketonuria, familial neurohypophyseal diabetes insipidus, and shortchain acyl-CoA

dehydrogenase deficiency and others. Depending on the protein, its misfolded conformation or appearance of aggregates may have variable impacts on cellular function. The downstream pathological events triggered by unfolded proteins include toxic effects such as: Ca^{2+} dysregulation (LaFerla, 2002) (Demuro et al., 2005), ER stress induction (Lindholm et al., 2006), production of ROS (Kadowaki et al., 2005) (Zhu et al., 2007) and induction of apoptotic responses leading to cell death (Stefani and Dobson, 2003) (Morishima et al., 2001) (Bucciantini et al., 2005). Additionally, most protein conformational diseases are accompanied by inflammatory responses (Manuelidis et al., 1997) (Casserly and Topol, 2004) (Williams and Nadler, 2007) (Aguzzi and Heikenwalder, 2006). Particularly, in patients with Alzheimers disease, release of proinflammatory mediators such as ROS, cytokines including IL-1, IL-6, TNF- α , IL-8, TGF- β , and macrophage inflammatory protein-1 α (MIP-1 α), and free radicals were detected at the site of accumulating misfolded molecules (Griffin et al., 1998) (Akiyama et al., 2000) (Finch and Morgan, 2007) (Heneka and O'Banion, 2007).

According to the energy landscape theory proposed by Bryngelson and Onuchic (Bryngelson et al., 1995) unfolded protein has the higher energy level (thermodynamically they possess high degrees of conformational entropy) in comparison with the fully folded, native conformation. As unfolded proteins try to attain an energy minimum and more stability, they thus tend to aggregate. Protein aggregation occurs through different intermediate states and can result in different structural appearances from unordered amorphous aggregates to highly ordered fibrils. One such organised form of protein aggregates, that has

been implicated in pathogenesis of several conformational diseases, are amyloids. They are linear unbranched proteins or protein fibrils whose formation is reversible despite their very stable structure (Wetzel, 2006). Amyloids share specific secondary composition i.e cross- β -structure (Meredith, 2005) however, the reciprocal arrangement of β -strands may vary hence amyloids can adopt more than one tertiary and quaternary structure (Jenkins and Pickersgill, 2001) (Chan et al., 2005b) (Roher et al., 2000). Amyloidosis arises mainly from mutations in the genes of plasma proteins that lead to their aggregation either systemically or locally in specific organs or tissues (Merlini and Bellotti, 2003). Amyloids have been associated with the pathology of several human diseases wherein abnormal deposition of amyloid fibrils in various organs may lead to amyloidosis or various disorders including neurodegenerative disease. An example of common amyloidosis is primary systemic amyloidosis (Gertz and Rajkumar, 2002) caused by amyloids formed by immunoglobulin light chain produced by plasma cells. Aggregates of Ig light chain or its fragments may accumulate in all organs of the body leading to several disorders such as congestive cardiac and renal failure, skin lesions or neuropathy.

The first described protein conformational diseases were neurodegenerative diseases. For example Alzheimer's disease (AD) is characterised by the formation of senile plaques of amyloid β -peptide (Mattson et al., 1998) (Pereira et al., 2004) (Roychaudhuri et al., 2009) (Lindholm et al., 2006) (Zhang et al., 2005). Disease pathogenesis is the consequence of extracellular accumulation of amyloid β -peptide and formation of filamentous intracellular structures of the tau protein within neurons (Roberts, 1988) (Rizzo et al., 2008) (Schenk et al.,

1999) (Lee et al., 2001). Hardy et al. proposed that accumulation of β -amyloid plaques in brain has a neurotoxic effect (Hardy and Allsop, 1991) since it can trigger production of high amounts of ROS with the impairment of the mitochondrial redox activity (LaFerla, 2002) (Kadowaki et al., 2005). Several reports implicate the UPR and ER stress in the cytotoxicity of amyloid β -peptide (Nakagawa et al., 2000). Upregulation of Bip/Grp78 and PERK in AD brain have been observed (Hoozemans et al., 2005). Furthermore, the UPR seems to account for neuronal cell death since in the absence of caspase-12, an effector of ER stress mediated apoptosis, cells shows reduced susceptibility towards ER stress and the abrogation of β -amyloid peptide-induced apoptosis in cortical neurons (Nakagawa et al., 2000) (Hitomi et al., 2004) (Ferreiro et al., 2006).

Taken together, amyloids and AD but also other debilitating conditions such as diabetes mellitus, vascular inflammation or atherosclerosis are associated with protein misfolding and subsequent protein aggregation. Therefore, these diseases can be categorised as conformational diseases for which, despite intensive studies, no sufficient cure is available yet.

1.4 Ankylosing Spondylitis

1.4.1 Clinical features

Ankylosing Spondylitis (AS) is a major subtype of the spondyloarthropathies (SpA), which are a group of interrelated rheumatic diseases that are characterized by common clinical symptoms and genetic predisposition. Beside AS, the other diseases belonging to the SpA clinical group are psoriatic

spondyloarthritis (PsA), reactive spondyloarthritis (ReA), spondyloarthritis associated with inflammatory bowel disease (IBD) and undifferentiated spondyloarthritis. The most common and significant clinical features are inflammatory back pain, asymmetric peripheral oligoarthritis mainly of the lower limbs, which serves as an important distinguishing factor with rheumatoid arthritis, psoriasis, chronic inflammatory bowel disease, enthesitis and anterior uveitis (Braun and Sieper, 2007). They are characterised by the absence of rheumatoid factor indicating a different pathophysiological mechanism of disease than that seen in rheumatoid arthritis.

AS is the most frequent and has the most severe course among SpA subtypes (Braun et al., 1998). The lower back pain caused by sacroiliitis is the most common clinical presentation of the disease and it improves with exercise (Sampaio-Barros et al., 2001) (Mansour et al., 2007). Characteristic symptoms of ankylosing spondylitis are spinal stiffness and loss of spinal mobility, that are caused by spinal inflammation and/or structural damage (Wanders et al., 2005). This is accompanied by chronic pain in the lower part of the spine or the entire spine. AS patients are particularly prone to develop syndesmophytes which are bony growths originating inside ligaments, as well as ankylosis which is the fusion of the bones of a joint. These structural changes are caused by osteoproliferation rather than osteodestruction. The peripheral arthritis mainly affects lower limbs (Dougados et al., 1991) but can also affect hip and shoulder joints in around 20% of AS patients. Enthesitis, the inflammation of enthesal sites, may also take place at many locations, including the spine (McGonagle et al., 1998) (Laloux et al., 2001).

The extraskeletal AS manifestations include anterior uveitis, psoriasis, colitis and the involvement of other organs such as the lung or heart. Eye inflammation seen in up to 40% of patients, is mainly restricted to the uvea and usually occurs unilaterally, however it can switch from one side to the other (Martin et al., 2002). Psoriasis, the inflammatory skin disease, commonly develops in AS patients. It leads to psoriatic nail lesions and/or inflammation of an entire digit. Inflammation within the gastrointestinal tract develops in around a quarter to half of AS patients whilst 5-10% of patients with AS reveal clinically overt inflammatory bowel disease as either a form of Crohn's disease or ulcerative colitis (Leirisalo-Repo et al., 1994) (de Vlam et al., 2000) (Rudwaleit and Baeten, 2006). However, both symptoms associated with psoriasis and inflammatory bowel disease can be regarded as either AS disease manifestations but also as basic subtype-defining entities with different genetics. Pulmonary and cardiovascular disease are rare manifestations of AS but the incidence of these features rise with increasing disease duration (Lautermann and Braun, 2002) (Mansour et al., 2007).

It was estimated that the number of new AS cases among adults in the UK would reach 4,400 per year per 100,000 people. AS characteristically affects young adults with an average age of onset between 20 and 30 years. Males are approximately twice as commonly affected as females, with the disease usually being more severe in men than women. Risk of developing AS is largely genetically determined as confirmed by twin studies which estimated AS heritability as >90% (Brown et al., 1997) (Pedersen and Mortensen, 2006).

1.4.2 The HLA-B27 association

The association between disease and the possession of the MHC class I allele HLA-B27 was firstly discovered in 1973 by Brewerton et al. There is a strong genetic association between AS and HLA-B27, since around 90% of AS patients are HLA-B27 positive (Table 1). The overall contribution of HLA-B27 to AS susceptibility is estimated to be between 20-40%. HLA-B27 is also present in around 50-70% of patients with other forms of SpA including psoriatic SpA, reactive arthritis, inflammatory bowel disease and isolated acute anterior uveitis in comparison with 7-8% of the general population.

However, the prevalence of HLA-B27 varies in population groups worldwide (Table 1), from 0% in African Bantu and Australian Aborigines to 50% in Native Americans (Khan, 2008). In Western European countries prevalence ranges from 3% to 18%. However, less than 5% of B27 positive individuals develop SpA and AS can also develop in B27 negative patients, suggesting the involvement of other genes in disease pathogenesis.

HLA-B27 distribution and strength of association varies not only with different forms of spondyloarthropathies, but also among different racial and ethnic populations as presented from some population in Table 1 (Boyer et al., 1997) (Lopez-Larrea et al., 2002) (Yamaguchi et al., 1995) (Madhavan et al., 2002) (Ayed et al., 2004) (Paladini et al., 2005) (Feltkamp et al., 2001) (Peschken et al., 1999) (Yang et al., 2013) (Gonzalez-Roces et al., 1997). Only approx. 8% of Northern Europeans (Caucasians) population possesses HLA-B27, but more than 90% of the patients with AS possess this gene. In contrast, among African Americans 2-4% of the general population has HLA-B27 whereas only 50- 60%

Table 1. HLA-B27 prevalence

Prevalence of HLA-B27 in disease^a			
Spondyloarthropathy			
Ankylosing Spondylitis	~ 90%		
Reactive Arthritis	40- 80%		
Psoriatic spondylitis	40-50%		
Enteropathic spondylitis	~ 75%		
IBD spondylitis	30-70%		
Prevalence of HLA-B27 in certain populations^{ab}			
	Healthy Population	AS patients	
		Strenght of Association of HLA-B27 with AS	Strenght of Association of HLA-B*2705 (OR) with AS ^b
General Population	~8%	90%	1.13
Caucasian	~8%	95%	1.54
Arab	3-5%	56%	
Sardinians	5%	70%	
Native Americans	20-45%	80 %	1.94
African Americans	~4%	50-60%	
Africans (West)	2-10%	6%	2.83
Asian			0.81
Japanese	1%	83%	
Southern Indian	1,5 %	76%	
Tunisians	6%	47%	
Chinese	8%	94%	
Indonesian	10%	40%	
Australian Aborigines	0%	-	-

OR – odds ration; ^a (Khan, 2008); ^b (Yang et al., 2013).

of AS patients are B27-positive. Interestingly, HLA-B27 allele was found to be present in 40% of native Indonesian AS patients, but in the same country it was found in 94% of Chinese patients with AS despite than the overall prevalence of HLA-B27 in latter population is lower (Feltkamp et al., 2001) (Mardjuadi et al., 1999).

1.4.2.1 HLA-B27 subtypes

There are more than 30 subtypes of HLA-B27. HLA-B*27:05 is strongly associated with the risk of AS and is the most widespread (Ball and Khan, 2001). The other subtypes seem to have evolved from HLA-B*27:05 by one or more amino acid substitutions (Khan, 1988a) (Khan, 1988b) (Khan and Ball, 2002). Other B27 alleles found to be associated with AS include B*27:01, B*27:02, B*27:03, B*27:04, B*27:07, B*27:08, B*27:10, B*27:14, B*27:15, B*27:19 (MacLean et al., 1993) (Armas et al., 1999) (Lopez-Larrea et al., 1995) (Gonzalez-Roces et al., 1997) (Garcia et al., 1998) (Garcia-Fernandez et al., 2001) (Tamouza et al., 2001). Two subtypes, B*27:06 and B*27:09, that are common in South Asia and Sardinia respectively, do not seem to predispose to AS development (Ball and Khan, 2001). However, cases of AS among B*27:06 and B*27:09 carriers were reported suggesting that these subtypes might not be completely protective for the disease (Gonzalez-Roces et al., 1997) (Olivieri et al., 2007) (Cauli et al., 2007) (Hou et al., 2007). Alternatively, there were other plausible explanations for disease development in these individuals as they were carrying the HLA-B*14:03 allele, which also exhibits an association with AS (Olivieri et al., 2007) (Cauli et al., 2007). Hence it seems that the MHC haplotype might be more significant for disease predisposition than the immunobiological differences between B*27:05 and B*27:09. Currently, B*27:06 and B*27:09 seem neither to predispose to AS nor prevent the occurrence of AS (Taurog, 2007).

Most of the differences between the B27 subtypes are clustered in the C/F pocket that binds the C-terminal peptide anchor residue (P Ω) which affects the

specific binding of antigenic peptides. Both HLA-B*27:05 and HLA-B*27:04 that are strongly associated with AS, express histidine at p114 and an aspartic acid at p116. HLA-B*27:09, B*27:06 and B*27:07 which can exhibit little or no correlation with the disease (Cauli et al., 2007) (Gomez et al., 2006) (Olivieri et al., 2007) (Ramos et al., 2002b) (Varnavidou-Nicolaidou et al., 2004) possess different sets of amino acids within this region. HLA-B*27:06 expresses aspartic acid and tyrosine while HLA-B*27:07 expresses asparagine and tyrosine at p114 and p116 respectively. HLA-B*27:09 differs by only a single amino acid, an aspartic acid-to-histidine substitution at p116 in comparison with HLA-B*27:05.

As the polymorphism between HLA-B27 subtypes is mainly clustered within the F pocket region, differences in the peptide repertoire are demonstrated at the C terminus of HLA-B27 binding peptides but also some subtle modulation at other positions exists. Almost all B27 subtypes require Arg at position 2 (R2) which is accommodated by the B pocket which is sterically and electrostatically ideal for binding arginine. Sequence analysis of peptides revealed that both B*27:06 and B*27:09, in comparison with disease-associated subtypes, have almost absolute requirement for nonpolar residues, including aliphatic and phenylalanine at the C-terminus (Fiorillo et al., 1997) (Sesma et al., 2002) (Ramos et al., 2002a). However, despite the polymorphism at p114/p116 there is a large overlap in the peptide repertoire between different subtypes. Mass spectrometry analysis revealed that peptides bound by HLA-B*27:05 and HLA-B*27:09 overlap by approximately 80% (Ramos et al., 2002a).

Despite polymorphisms within the peptide binding groove influencing peptide presentation and T cell repertoire selection (Fiorillo et al., 2000) (Chen et al., 1993) (Luz et al., 2002) (Sesma et al., 2002), most of the data indicate that the association of HLA-B27 subtypes with AS does not correlate with common peptide presentation among disease-associated subtypes or with obvious peptide motifs (Gomez et al., 2006).

However subtype polymorphisms within the peptide binding groove were found to influence other features of MHC class I biology such as the tendency to misfold both within the ER and at the cell surface (Mear et al., 1999) (Galocha and de Castro, 2008) (Goodall et al., 2006) These features will be discussed in 3.3 Results chapter.

1.4.2.2 SpA animal models

Initial approaches in generating AS animal models were unsuccessful since there was no development of inflammatory arthritis in mice in which HLA-B27/h β 2m transgenes were expressed (Kievits et al., 1987) (Krimpenfort et al., 1987). This could simply be accounted for by the genetic background of the mice utilised in these studies since later experiments with different mouse strains allowed for the generation of disease prone transgenic animals (Weinreich et al., 1995a) (Khare et al., 1998) (Kingsbury et al., 2000). Particularly, in the B27-associated joint disease, mouse Ankylosing enthesopathy (ANKENT), the H-2^k haplotype seemed to be a crucial risk factor (Weinreich et al., 1995a) (Capkova and Ivanyi, 1992). Although, ANKENT can occur also in non-transgenic mice (Capkova and Ivanyi, 1992), the expression of HLA-B27 significantly increased the frequency of joint ankylosis (Weinreich

et al., 1995a) (Weinreich et al., 1995b) (Weinreich et al., 1997). Despite these observations, ANKENT does not parallel human AS. ANKENT resembles AS in its dependency on age, gender and presence of non-MHC as well as MHC risk factors and like AS is more prevalent among young males that express HLA-B27 (Weinreich et al., 1995b).

Another mouse B27-associated arthropathy that has been reported is spontaneous inflammatory arthritis (SA) (Khare et al., 1998). In these HLA-B27 transgenic mouse models, an SpA phenotype involving the joints of the digits, feet and ankles and dystrophic nail changes, was observed when the endogenous gene for B2m was deleted (Khare et al., 1995) (Khare et al., 1996) (Khare et al., 1998). Similarly to ANKENT, SA disease development was also influenced by genetic background in these studies (Kingsbury et al., 2000). However, ANKENT and mouse SA stand at the opposite ends for the dependence on endogenous β 2m since B2m-depletion actually reduces the incidence of ANKENT (Weinreich et al., 1997). Hence, there might be fundamental difference in a B27-mediated mechanism of disease development in these two models.

In order to address the requirement of β 2m in inflammatory arthritic disease development in B27 transgenic mouse models Colbert and colleagues examined mice of several genetic backgrounds deficient in β 2m and TAP. They found that β 2m and TAP deficiency alone was sufficient to cause development of spontaneous arthritis regardless of HLA-B27 expression (Kingsbury et al., 2000). Although, mice exhibited a disease phenotype independently of HLA-

B27, these studies emphasized the idea of MHC misfolding in the development of an arthritic phenotype (Kingsbury et al., 2000).

The demonstration that there is a direct role for HLA-B27 in AS has come from studies of transgenic rats expressing more than 50 gene copy numbers of HLA-B27, which develop a multisystemic inflammatory disease with similar characteristics to human SpAs (Hammer et al., 1990). However, in this model, transgenic rats do not fully acquire a disease that resembles human AS, as there is a reduced incidence of ankylosis of the axial skeleton, whilst the inflammatory bowel conditions are much more prominent than those observed in human AS patients. Subsequent studies have revealed that by further increasing the expression of hB2m in B27/hB2m transgenic rats, this alters the disease phenotype and result in more severe arthritis and significant axial disease with the reduction in accompanying inflammatory bowel disease (Tran et al., 2006).

Furthermore, the disease phenotype requires high, non-physiological copy numbers of the B27 transgene in order to develop. Importantly, disease development is not a non-specific result of MHC class I misfolding since disease did not occur in rats overexpressing another transgene, HLA-B*0702/h β 2m, confirming the specificity of B27-mediated inflammatory disease (Taurog et al., 1999). Interestingly, elevated level of HLA-B27 on peripheral blood mononuclear cells in AS patients, versus healthy B27-positive individuals has been observed (Cauli et al., 2002). Since the level of other HLA molecules in these AS patients was not upregulated it strongly suggested that high levels of B27 expression may be a contributing factor to disease. Furthermore, disease development was also found to be dependent on the rat strain (most

backgrounds tested, Lewis, Fisher, and PVG, are permissive whilst the Dark agouti strain is resistant) indicating that similarly to the B27-transgenic mouse models, genetic background is important, therefore emphasizing the involvement of other genes in AS development.

The other contributing factor to AS development in the transgenic B27 rat model is exposure to gastrointestinal microbes. When raised under entirely germ-free conditions, HLA-B27/h β 2m expressing rats did not develop a disease phenotype (Taurog et al., 1994). However, presence of normal gut flora, such as *Bacteroides* spp., was sufficient to elicit gut inflammation and arthritis (Taurog et al., 1994) (Rath et al., 1996) (Rath et al., 1999). Similarly, mice expressing the human HLA-B27 transgene did not develop any spontaneous disease, unless they were exposed to *Yersinia enterocolitica* (Nickerson et al., 1990b) (Nickerson et al., 1990a). The latter is a potentially useful experimental model for human HLA-B27 associated Reactive Arthritis (ReA) since it was found that several gastrointestinal or genito-urinal pathogens led to disease development in ReA patients (Colmegna et al., 2004). Furthermore, enteric bacteria seem to be implicated in triggering SpA-like disease since subclinical intestinal inflammation has been reported in patients with SpA (De Keyser et al., 1998) (Lionetti et al., 2000). Although gut inflammation seems to be an important risk factor for clinically overt AS, the exact mechanisms by which mucosal inflammation might lead to disease remains elusive.

Taken together, despite the phenotypic differences in comparison with human SpA, HLA-B27-transgenic animal models emphasize the importance of MHC

class I proteins in the pathogenesis of arthritis and provide an insight into etiological, environmental, and genetic risk factors.

1.4.2.3 Arthritogenic peptide and molecular mimicry hypothesis

Several theories have been proposed with regard to the molecular pathogenic role of HLA-B27 in AS development and pathogenesis. The earliest theories emphasized the HLA-B27 peptide-presenting specificity in disease pathogenesis. Initially it was proposed that there must exist one or more specific peptide(s) for HLA-B27 that can trigger a CTL response. In this theory, chronic inflammatory disease results from autoreactive T-cell mediated cytotoxicity. In support of this, CD8⁺ T-cells reacting with collagen-derived self-peptides in a B27-dependent manner were identified in the synovial fluid of AS patients (Atagunduz et al., 2005). It was proposed that cartilage-directed cellular autoimmunity may be involved in joint-specific tissue damage in patients with AS, however, the pathogenic relevance of this peptide has not been confirmed.

Related hypotheses postulated that HLA-B27 might present self-antigens that by mimicking foreign peptides derived from either intracellular bacteria or viruses, could activate autoreactive T lymphocytes (Breban et al., 1993). In support of this “molecular mimicry” hypothesis, HLA-B27-restricted CD8⁺ T-cell clones with specificity for bacteria have been identified in the synovium and peripheral blood of ReA and AS patients (Hermann et al., 1993). Although HLA-B27-binding *Yersinia* and *Chlamydia*-derived peptides that could stimulate CD8⁺T cell response in synovial fluid were identified (Ugrinovic et al., 1997)

(Kuon et al., 2001), no self-peptide resembling these antigens has been identified so far.

Another findings supporting the molecular mimicry hypothesis relates to the conformation of peptides which bind to HLA-B27. Some self-peptides were found to display conformational dimorphism when bound to HLA-B27 molecules (Fiorillo et al., 2005) (Hulsmeyer et al., 2004) (Ruckert et al., 2006). A self-peptide derived from vasoactive intestinal peptide receptor 1 (VIPR1) resembling the sequence of an Epstein-Barr virus-derived epitope, was found to be presented by HLA-B27. Although VIPR1 can be presented by both HLA-B*27:05 and 09 subtypes, it can exclusively bind to HLA-B*27:05 in a non-conventional conformation (Hulsmeyer et al., 2004). Similarly, a pLMP2 peptide derived from Latent membrane protein 2 of EBV was shown to be presented by B*27:05 and B*27:09 subtypes in two drastically different conformations (Fiorillo et al., 2005). Whether this alternative conformation can elicit specific CTL response has not been formally tested. Functional studies revealed that B*27:09 individuals very rarely possess pVIPR-reactive CTLs, whereas such T cells are frequently observed in peripheral blood of patients with AS, although they occur also in healthy B*27:05 individuals (Fiorillo et al., 2000). However, Hulsmeyer demonstrated that the majority of the 39 CTL cell lines tested were able to lyse both B*27:05 and B*27:09 presenting pVIPR with similar efficiency and only one CTL cell line was reactive exclusively to HLA-B*27:05-pVIPR expressing cells (Hulsmeyer et al., 2004). It is possible that this CTL cell line was directed against a alternative peptide structure presented exclusively by B*27:05 (Hulsmeyer et al., 2004). It was subsequently proposed that

dual/alternative conformations could account for less efficient negative selection in the thymus and similarities between self and viral-peptides could lead to a cross-reactive CTL response by clones which have escaped elimination in the thymus. Furthermore, HLA-B27 restricted CD8⁺ T cell responses to this pVIPR peptide were more frequent in AS patients compared with healthy HLA-B27 positive controls and HLA-B*27:09 individuals (Fiorillo et al., 2000).

Another related hypothesis proposes that B27 itself serves as an auto-antigen due to its sequence homology with certain bacterial proteins (Boyle et al., 2001) (Jardetzky et al., 1991) (Schwimmbeck et al., 1987) (Lahesmaa et al., 1991). Particularly, a fragment derived from the cytoplasmic tail of HLA-B27 (p309-320) which mimicks protein sequences from *Chlamydia trachomatis*, was found to be naturally presented by B27 AS-associated subtypes including B*27:05, B*27:04 and B*27:02 but not B*27:06 and B*27:09 (Ramos et al., 2002a). Nevertheless, such a molecular-mimicry mechanism does not explain the tissue specificity observed in HLA-B27 associated diseases.

Despite the above observations supporting the molecular mimicry theory as a molecular mechanism for the involvement of HLA-B27 in AS pathogenesis, classical peptide presentation by HLA-B27 to cytotoxic CD8⁺ T cells may not be crucial in AS pathogenesis. Dorris et. al. have shown that transgenic rats with high expression of HLA-B27 still develop a multisystemic inflammatory disease that includes arthritis and colitis, despite depletion of CTLs (May et al., 2003). Moreover, HLA-B27-expressing bone marrow cells can transfer disease to wild type animals, indicating that HLA-B27 does not have to be expressed on non-haematopoietic cells, including thymic epithelial cells which are involved in T-

cell education (Breban et al., 1993). Furthermore, B27-transgenic mouse models of spontaneous arthritis develop disease in the absence of β 2m (Khare et al., 1995), suggesting that cell surface expression of stable MHC class I heavy chain- β 2m-peptide complexes are not required. Finally, both the large overlap in the peptide repertoire between AS-associated and non AS-associated B27 subtypes as well as minimal overlap between HLA-B*27:05 and other disease associated HLA alleles argues against a molecular mimicry based mechanism. Particularly, peptide binding analysis of the antigens eluted from HLA-B*27:05 and AS-associated HLA-B*14:03 revealed that they share only 3% of their peptide repertoire (Lopez-Larrea et al., 2002) (Diaz-Pena et al., 2008) (Merino et al., 2005). Taken together, most data would argue against, although do not exclude, a mechanism of spondyloarthritis mediated by specific peptide ligands.

1.4.2.4 Misfolding Hypothesis

1.4.2.4.1 Biochemical characteristics of HLA-B27

Recently, the tendency of HLA-B27 to misfold has been postulated as a new mechanism by which B27 may contribute to AS pathogenesis. HLA-B27 possesses unusual biochemical characteristics such as an enhanced tendency to misfold and form covalent heavy chain homodimers which were proposed to be involved in AS pathogenesis (Mear et al., 1999) (Colbert, 2000b) (Colbert, 2000a). Support for the misfolding hypothesis has come from both mouse and rat models. In the B27-transgenic mouse model, development of spontaneous

inflammatory arthritis is observed when MHC class I molecules are unable to assemble due to the lack of $\beta 2m$ or TAP expression (Khare et al., 1995) (Kingsbury et al., 2000). Furthermore, in the transgenic-rat model, only high levels of the HLA-B27 transgene lead to consistent and reproducible disease. Such high levels of HLA-B27 expression can potentially lead to heavy chain misfolding and trigger development of the disease (Taurog et al., 1993).

HLA-B27 was found to exhibit an enhanced tendency to undergo ERAD (Mear et al., 1999), the pathway by which misfolded MHC class I molecules are disposed of from the ER (Hughes et al., 1997). Mear et al. demonstrated that despite an intact assembly and antigen processing pathway, HLA-B27 was susceptible to misfolding (Mear et al., 1999). This also correlated with the slow folding kinetics of HLA-B27, which seems to be strongly influenced by the composition of the B pocket of the peptide binding groove (Mear et al., 1999) (Dangoria et al., 2002). The combination of residues within the B pocket including His9, Thr24, Glu45, Cys67, Lys70, Ala71 and Gln97 is characteristic of HLA-B27 when compared with other B alleles. Although, it would be tempting to assume that this observation could also support the “arthritogenic peptide” hypothesis, introduction of these residues into non-SpA associated HLA molecules resulted in enhanced misfolding (Mear et al., 1999) (Antoniou et al., 2004).

The other unusual characteristic of HLA-B27 is its ability to form aberrant disulphide linked heavy chain dimers. Initially, HLA-B27 aggregates were observed following purification of recombinant HLA-B27 (Allen et al., 1999), but were soon confirmed in *in vivo* studies by several laboratories. Originally

discovered by Allen and colleagues, HLA-B27 dimers were thought to lack β 2m (Allen et al., 1999). However, later studies revealed that HLA-B27 can form two types of aberrant disulphide-linked complexes based on recognition by conformation and non-conformationally-specific antibodies (Dangoria et al., 2002) (Bird et al., 2003). B27 homodimers associated with β 2m could be detected with the W6/32 antibody which recognises fully folded β 2m associated molecules (Barnstable et al., 1978), whilst β 2m-free heavy chain dimers could be recognised by the HC10 antibody which is specific for partially folded/unfolded heavy chain molecules lacking β 2m and peptide (Stam et al., 1986). Furthermore, several *in vivo* analyses report that HLA-B27 unfolded dimers are not homogenous since they can be detected as several distinct populations (Dangoria et al., 2002) (Bird et al., 2003) (Tran et al., 2004) (Antoniou et al., 2004). Nevertheless, how these different B27 heavy chain dimers form and their role in disease pathogenesis is unknown.

HLA-B27 aberrant disulphide linked dimers have been detected both within the ER and at the cell surface (Dangoria et al., 2002) (Antoniou et al., 2004) (Bird et al., 2003) (Kollnberger et al., 2004). It seems that ER resident dimers do not contribute to the cell surface population since they do not traffic through the secretory pathway (Dangoria et al., 2002). The ER dimer population comprises of approximately 25% of the newly synthesized HLA-B27 heavy chain and are predominantly β 2m-free as determined by their HC10 reactivity (Dangoria et al., 2002). ER resident dimer formation seems to be influenced by the maturation kinetics of the heavy chain (Antoniou et al., 2004). It was shown that even non-disease-associated HLA class I alleles, such as HLA-A2, can be induced to

form heavy chain dimers by temperature-mediated slowing of the assembly rate (Antoniou et al., 2004). Furthermore, in β 2m and tapasin-deficient cell lines populations of unfolded HLA-B27 dimers accumulate, confirming their misfolding nature (Dangoria et al., 2002). On the contrary, fully folded W6/32-reactive HLA-B27 dimers have not been detected within the ER so far.

In comparison with ER resident homodimers, there is evidence for the presence of both HLA-B27 β 2m-free as well as β 2m-associated heavy chain dimers at the cell surface although the latter is much more abundant (Dangoria et al., 2002). The formation of β 2m-associated B27 dimers at the cell surface is elusive however their appearance is determined by the intact assembly and peptide acquisition pathway since in the absence of TAP, tapasin and β 2m the pool of W6/32-reactive B27 dimers is ablated (Dangoria et al., 2002). HLA-B27 β 2m-free dimers are thought to form during endosomal recycling and consist of 6% of the total MHC class I heavy chain (Allen et al., 1999) (Bird et al., 2003). Experiments with inhibitors of endosomal acidification indicate that HLA-B27 expressed as a heterodimer of class I heavy chain- β 2m can enter the endosomal pathway where, due to low endosomal pH, the β 2m subunit dissociates and β 2m-free heavy chains can be recycled to the cell surface (Bird et al., 2003). The population of unfolded cell surface B27 dimers increases in the absence of tapasin indicating that instability of MHC class I molecules at the cell surface accelerates dimer formation (Dangoria et al., 2002).

The β 2m-free HLA-B27 at the cell surface is not a homogeneous population. A small percentage of cell surface HLA-B27-peptide complexes are recognized by the monoclonal antibody MARB4 (Urban et al., 1994). The full characterisation

of the MARB4-reactive subset of HLA-B27 molecules, revealed that they lacked β 2m, but stably associated with peptide (Malik et al., 2002). Dangoria et al. also showed that the majority of MARB4-reactive B27 heavy chains form dimers indicating that the MARB Ab binds to β 2m-free, peptide containing HLA-B27 homodimers at the cell surface (Dangoria et al., 2002). Taken together, these observations reveal that HLA-B27 at the cell surface can exist as β 2m-associated/ β 2m-free monomers and dimers and all these forms can exist as peptide-receptive or peptide-associated forms. The plausible implication of these forms in AS pathogenesis will be further discussed.

1.4.2.4.2 Intracellular HLA-B27 misfolding and AS pathogenesis

One of the consequences of misfolded HLA-B27 protein within the ER is the induction of ER stress by triggering the UPR (Figure 1.6) (Turner et al., 2005) (Turner et al., 2007). The UPR is characterised by increased expression of the ER resident chaperone BiP which was found to persist on misfolded and oligomeric HLA-B27 molecules (Antoniou et al., 2004) (Tran et al., 2004) and this prolonged interaction could lead to activation of the UPR signalling pathway. This in turn can disrupt cellular function and may lead to the release of proinflammatory cytokines (Colbert, 2000b).

Direct evidence suggesting that HLA-B27 can mediate UPR activation during disease development has come from studies by Turner et al. It was reported that bone marrow-derived macrophages (BMDM) from HLA-B27 transgenic rats exhibiting inflammatory arthritis, showed evident activation of the UPR as measured by the upregulation of the UPR effector protein XBP-1s (Turner et al., 2005) (Turner et al., 2007). The UPR was also detected in inflamed tissue

within the gastrointestinal track but not in cells from the spleen or thymus indicating that UPR activation was not widespread in these animals (Turner et al., 2005) (Turner et al., 2007). Moreover, INF- γ -mediated cell activation of BMDM B27-expressing cells resulted in HLA-B27 upregulation and increased HLA-B27 misfolding as measured by the upregulation of HC10-reactive heavy chain as well as enhanced detection of high Mw species and prolonged associations with BiP. This correlated with UPR activation whilst similar treatment of cells from HLA-B7-expressing rats, though resulting in class I heavy chain upregulation, did not trigger the UPR, suggesting that UPR activation is relatively specific to HLA-B27 misfolding rather than general MHC class I misfolding (Turner et al., 2007).

The idea that aggregation of misfolded HLA-B27 molecules participates in the pathogenesis of B27-associated disease was supported by studies by Tran et al, who reported the presence of HLA-B27 heavy chain dimers in B27-transgenic disease prone rats (Tran et al., 2004). In a consecutive study, in order to “improve the folding” of HLA-B27 in rats expressing high copy numbers of the B27 transgene, Tran et al. introduced an additional 35 copies of h β 2m by crossbreeding (Tran et al., 2006). Surprisingly, the additional β 2m and improved folding of HLA-B27 led to the exacerbation of skeletal disease but prevented colitis and gut inflammation. It was also proposed by the authors that this correlated with reduced HLA-B27 misfolding as assessed by a reduction in the proportion of unfolded HC10-reactive to folded heavy chain as well as accelerated maturation kinetics. This was also accompanied by diminished UPR triggering as demonstrated by a 30% reduction in the level of BiP mRNA in

splenocytes (Tran et al., 2006). It was suggested that B27 misfolding might participate in distinct phases of SpA development or that the skeletal and gut diseases may indeed be distinct. Although the authors concluded that B27 misfolding might not be critical in development of arthropathy, whether the additional h β 2m directly prevented UPR activation, was not tested.

Although independently of HLA-B27, the importance of MHC class I misfolding in disease development was also highlighted in transgenic mouse models (Kingsbury et al., 2000). Colbert et al. observed that disrupting the MHC class I assembly pathway by knocking out β 2m or TAP resulted in the development of spontaneous arthritis (Kingsbury et al., 2000). Nevertheless, there is a strict but specific correlation between HLA-B27 upregulation, and its misfolding, which can trigger the UPR in the transgenic rat model. Whether UPR is activated in human cells during AS development has not been determined but interestingly elevated level of HLA-B27 molecules have been reported in AS patients (Cauli et al., 2002).

A potential link between HLA-B27 misfolding and triggering of the UPR leading to inflammation could potentially involve activation of the transcription factor NF- κ B (Pahl and Baeuerle, 1995). The pharmacologically-induced accumulation of misfolded molecules within the ER was shown to activate NF- κ B, which can target proinflammatory cytokine genes including IL-6 and TNF- α (Figure 1.6) (Li et al., 2005). In turn, IL-6, TNF- α and IFN- γ were reported to augment ER stress responses, particularly by induction of the transcription factor CREBH (Zhang et al., 2006a). CREBH is mainly induced in hepatocytes where it was reported to mediate the acute phase response and lead to enhanced production of

proinflammatory mediators (Zhang and Kaufman, 2008). Taken together, these observations suggest that there might be some positive feedback in which proinflammatory cytokines and UPR exacerbate inflammation (Xue et al., 2005) (Hu et al., 2006). Indeed, synergistic induction of the proinflammatory cytokine IFN- γ was observed in HLA-B27/human β 2m-transgenic rat macrophages undergoing a UPR as result of HLA-B27 up-regulation (Smith et al., 2008). This cytokine has several immunoregulatory effects including activation of NK cells and macrophages, promoting survival of T cells and maturation of DCs linking together innate and adaptive immune responses (Marrack et al., 1999) (Biron, 2001) (Le Bon and Tough, 2002) (Theofilopoulos et al., 2005). Furthermore, IFN- γ is an important regulator of bone homeostasis and formation through inhibition of osteoclast differentiation (Takayanagi et al., 2002).

The misfolding hypothesis could to some extent, explain the differential association of HLA-B27 subtypes to AS. The non-AS associated subtypes, HLA-B*27:06 and 09, were found to be less susceptible to misfolding than the disease associated subtypes such as B*27:02, B*27:04 and B*27:05, which is indicative of a relationship between folding properties and disease development (Goodall et al., 2006) (Lopez de Castro, 2007) (Galocha and de Castro, 2008) (Fussell et al., 2008). However, there is an incomplete correlation between HLA-B27 subtype folding and AS. The HLA-B*27:07 subtype, which exhibit both positive and negative associations with disease, folds with similar efficiency to non-AS-associated subtypes since it has a similar export rate from the ER and a minimal ratio of unfolded (HC-10-reactive) to fully folded (ME1-reactive) material which did not increase even upon IFN- γ -mediated MHC class

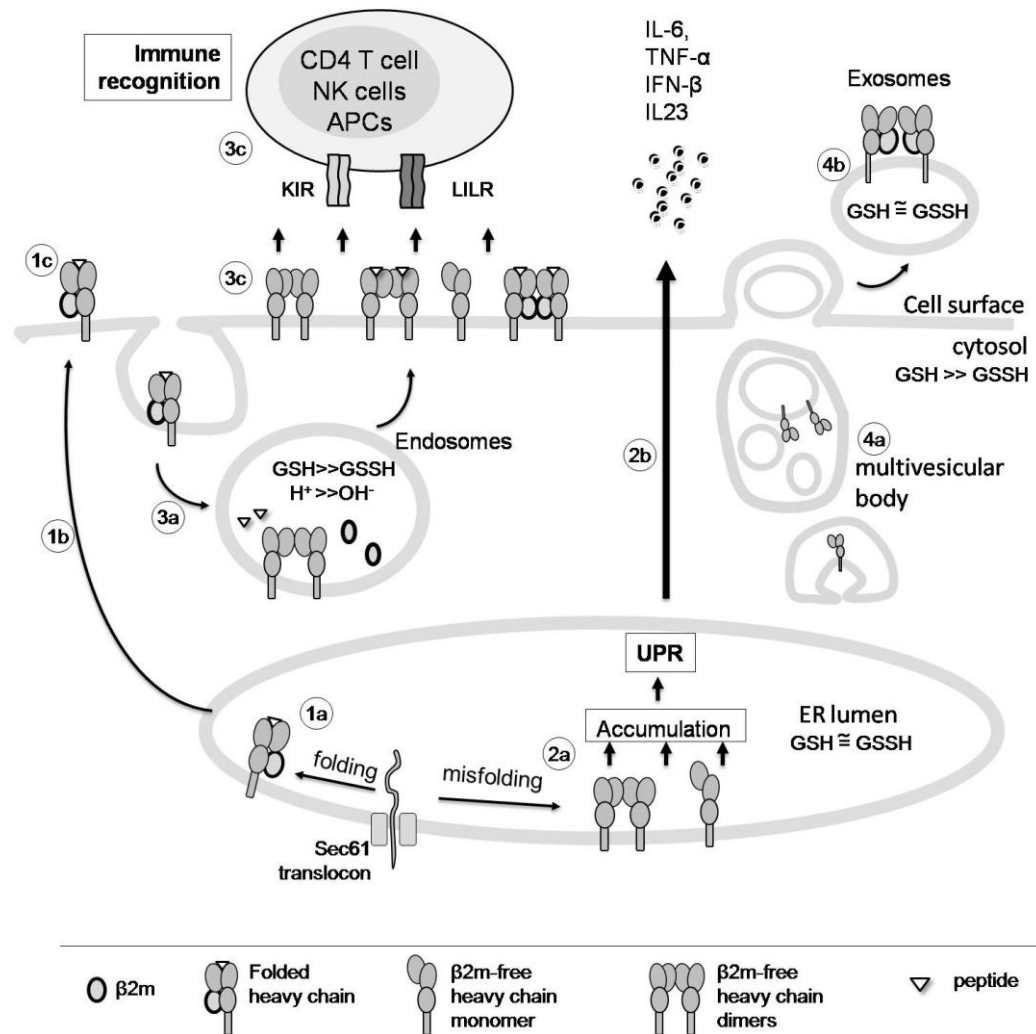


Figure 1.6. Aberrant HLA-B27 heavy chain forms and their contribution to AS. 1) Within the oxidizing environment of the ER (depicted by the reduced glutathione [GSH] to glutathione oxidized [GSSG] ratio) HLA-B27 heavy chain forms stable trimeric complexes with peptide (1a) which are transported to the cell surface (1b). 2) HLA-B27 can also misfold within the ER and form several heavy chain-dimer species (2a) that may accumulate and trigger UPR which in turn lead to upregulation of proinflammatory cytokines (2b). 3) HLA-B27 cell surface dimers form due to the internalization and recycling of fully folded heavy chain (1c) through the acidic and oxidizing environment of the endocytic pathway (3a). The aberrant cell surface HLA-B27 forms (3b) may mediate abnormal ligand interactions with KIR and LILR receptors on CD4⁺ T cells, Natural Killer (NK) cells or antigen-presenting cells (APCs) (3c). 4) β 2m-associated heavy chain dimers can be detected within exosomes, which are derived from multivesicular bodies (4a). The oxidising environment of the exosomes favours the disulphide bond formation between the cytoplasmic cysteines of two heavy chains (4b).

upregulation (Galocha and de Castro, 2008). Furthermore, analysis of folding properties of two differentially AS associated HLA-B*14:02 and B*14:03 alleles (Lopez-Larrea et al., 2002), which differ by 1 amino acid, incompletely supports the misfolding hypothesis as a mechanism underlying subtype association with AS (Merino et al., 2008).

The maturation rate, as determined by the half-life of Endo H-sensitive β 2m-free heavy chains as well as the stability of B*14:03 and B*14:02 were shown to be very similar and at the same time different from B*27:05. Thus, these features cannot account for the fact that only B*14:03 and B*27:05 allotypes are associated with AS (Merino et al., 2008).

Taken together, although not complete, there is a correlation, between inefficient heavy chain folding and subtype AS susceptibility, however this requires further investigation.

1.4.2.4.3 Aberrant cell surface HLA-B27 heavy chains

Both ER resident and cell surface dimeric or misfolded monomeric HLA-B27 forms were proposed to have the potential to be involved in SpA development (Figure 1.6). As mentioned previously, HLA-B27 aberrant β 2m-free monomeric forms have been detected at the cell surface of several cell types such as activated and resting T cells and EBV-transformed B cell lines (Schnabl et al., 1990) (Madrigal et al., 1991) (Rock et al., 1991) (Demaria et al., 1992) (Matko et al., 1994). Also HLA-B27 homodimers have been detected at the cell surface of lymphoblastoid cells, rat DC from the B27-transgenic rat model and HLA-B27 transfected cells (Allen et al., 1999) (Bird et al., 2003) (Kollnberger et al., 2002)

(Kollnberger et al., 2004). Hence it was proposed that, the expression of abnormal forms of HLA-B27 at the cell surface may have pathogenic roles in AS through the interaction with immunoregulatory receptors on NK and/or T cells including leukocyte immunoglobulin-like receptors (LILRs) and killer immunoglobulin-like receptors (KIRs) (Figure 1.6). In order to determine the potential consequences of HLA-B27 homodimeric forms on leukocyte function, binding of *in vitro* refolded β 2m-free HLA-B27 dimer complexes were tested on cell lines transfected with particular KIR, ILT or LIR molecules. It was demonstrated that immune receptors KIR3DL1, KIR3DL2, LIRB2 and LIRA1 are able to recognise the homodimeric forms of HLA-B27 (Allen et al., 2001) (Kollnberger et al., 2002). However, only KIR3DL2 was shown to bind exclusively to B27 β 2m-free heavy chain dimers but not β 2m-associated B27 monomeric molecules. Furthermore, although KIR recognition of HLA-B*27:05 heterodimers, like other HLA-A, -C and B alleles, is influenced by peptide sequence (Peruzzi et al., 1996a) (Peruzzi et al., 1996b) (Malnati et al., 1995) (Hansasuta et al., 2004) (Stewart et al., 2005a), binding of B27 β 2m-free homodimers to KIR3DL2 is independent of peptide (Kollnberger et al., 2007). Recognition of dimeric HLA-B27 molecules by KIR3DL2 may promote survival of KIR3DL2-expressing NK or T cells as demonstrated in *in vitro* studies in which KIR3DL2⁺ NK cells were protected from apoptosis when cocultured with cells expressing B27 dimers at the cell surface (Chan et al., 2005a). The interaction of KIR3DL2 with β 2m-free B27 homodimers inhibits production of IFN- γ by KIR3DL2-expressing NK or T cells (Kollnberger et al., 2007). Hence, B27 homodimer-KIR3DL2 interactions could promote survival of KIR3DL2-expressing self-reactive leukocytes in patients.

Furthermore, APCs expressing HLA-B27 dimers can stimulate the survival, proliferation, and IL-17 production of KIR3DL2⁺ CD4⁺ T cells. KIR3DL2⁺ CD4⁺ T cell lines from SpA patients but not from healthy controls or KIR3DL2⁻ CD4⁺ T cells, were found to secrete 4-fold more IL-17 (Bowness et al., 2011), a proinflammatory cytokine postulated to play an important role in AS pathogenesis (Wendling et al., 2007) (Wang et al., 2009) (Singh et al., 2007) (Melis et al., 2010).

Taken together, ligation of immune receptors by HLA-B27 β 2m-free dimers appears to promote the survival of KIR-expressing leukocytes and influence immune cytokine production which have been implicated in AS development and pathogenesis. The putative implications of the above data is supported by observations that KIR3DL2 was found to be upregulated on both NK and T-cells in the periphery and synovium in AS patients along with an expanded subset of KIR3DL2⁺ cells (Chan et al., 2005a).

Fabian et al. proposed an alternative mechanism to explain the altered HLA-B27-KIR interaction. Using an infrared spectroscopy approach, which monitors conformational changes within proteins or conformational differences between related proteins (Barth, 2007) Fabian et al reported that B*27:05 and B*27:09 differ in the flexibility of the α -helices surrounding the peptide binding groove (Fabian et al., 2010). HLA and KIR molecules typically behave as rigid bodies, in comparison with the more flexible HLA–TCR interactions (Boyington and Sun, 2002) (Rudolph et al., 2006). Furthermore, KIR recognition of HLA has previously been shown to be critically dependent on amino acid residues in the α 1 helix of class I heavy chains. Hence, elevated dynamics of the HLA-B*27:05

subtype in comparison with B*27:09 could alter interactions with KIR-expressing cells.

Since B27 transgenic nude rats which lack a thymus, remain healthy, suggest that T cells are also necessary in disease development (Breban et al., 1996). As mentioned before CD4⁺ T cells seem to be more crucial in AS development than CD8⁺ T cells. In the B27 transgenic rat model elimination of CD8 $\alpha\beta$ ⁺ T cells does not prevent development of SpA-like disease (May et al., 2003) whereas a population of CD4⁺ but not CD8⁺ T cells were found to be expanded in SpA patients (Chan et al., 2005a). It was then proposed that CD4⁺ T cell may also be able to recognise aberrant HLA-B27 forms. In support of latter, Boyle and colleagues demonstrated that CD4⁺ T cells could be expanded *in vitro* by stimulation with cell lines defective in antigen processing pathways and thus express misfolded forms of HLA-B27 (Boyle et al., 2001) (Boyle et al., 2004). Furthermore, in the B27-transgenic rat model, an antigen-independent defect in priming of naive CD4⁺ T lymphocytes by DCs was observed, which was thought to partially result from impaired formation of immunological synapses between DCs and T cells (Stagg et al., 1995) (Hacquard-Bouder et al., 2004) (Hacquard-Bouder et al., 2007). The latest studies demonstrate that expression of mature HLA-B27 on DC interferes with formation of an IS by impairing the engagement of costimulatory molecules (Hacquard-Bouder et al., 2007). Since costimulation is crucial in the generation and maintenance of regulatory T cells (Tang et al., 2003) (Liang et al., 2005) (Tai et al., 2005) it was suggested that the presence of B27-expressing DCs, could result in a failure to produce and/or maintain

tolerogenic CD4⁺CD25⁺ T cells, contributing to the expansion of pathogenic CD4⁺ T-cells (Stagg et al., 1995) (Hacquard-Bouder et al., 2007).

Taken together, these studies indicate that aberrant forms of HLA-B27 expressed at the cell surface can interact with several different immune receptors present on cells such as NK and CD4⁺ T-cells, which were found to be expanded during inflammation. Hence, altered immune recognition of HLA-B27 may be an important parameter in the pathogenesis of AS.

1.4.3 Other AS genetic associations

While HLA-B27 defines a large proportion of the genetic risk of AS, it is now well recognized, that other genes must be involved in disease development. The importance of additional genes in AS development was supported by twin and family studies in which <50% of the overall risk is due to HLA-B27 (Brown et al., 1997). Furthermore, only 5% of HLA-B27 positive people develop disease whilst in HLA-B27 positive individuals that have first-degree relatives with AS, the risk of disease development is 6-16 times higher in comparison with those without such a family history (Calin et al., 1983) (van der Linden et al., 1983). The existence of other genetic factors, is also supported by B27-transgenic animal models, in which disease development is highly dependent on the genetic background (Taurog et al., 1999) (Weinreich et al., 1995a) (Kingsbury et al., 2000).

1.4.3.1 Other MHC genes

Approximately 10% of Caucasian patients with AS are HLA-B27 negative suggesting there are other genetic associations potentially both within and

without the MHC region. There is much evidence supporting associations with other HLA-B and non-HLA-B alleles with AS (Yamaguchi et al., 1995) (Lopez-Larrea et al., 2002) (Merino et al., 2005).

Serologic HLA class I typing of Japanese patients with AS revealed that 3 of 8 HLA-B27-negative individuals were positive for HLA-39 (Yamaguchi et al., 1995). HLA-B*14:03 is found almost exclusively in African (Lopez-Larrea et al., 2002) (Ellis et al., 2000) and African American populations (Cao et al., 2001) (Tu et al., 2007). Since its prevalence is <1% and AS is also very rare in African populations (Mijiyawa et al., 2000), the finding that 4 out of 8 unrelated patients with AS from the sub-Saharan region and 2 out of the 3 from Zambia with AS who were B*14:03 positive supports the hypothesis that B*14:03 is a susceptibility factor for AS (Lopez-Larrea et al., 2002) (Diaz-Pena et al., 2008). Furthermore, HLA-B60 and HLA-B61 have been shown to be an increased risk factor in Caucasian, Taiwanese and Chinese AS patients (Wei et al., 2004) (Brown et al., 1996). The other MHC class I genes found to be associated with AS include B38, B40 and B52 (Madhavan et al., 2002). Nevertheless, none of these studies fulfil the criteria which would allow to fully classify the association of these genes with AS, that is: large sample sizes and control for linkage disequilibrium with known HLA associations.

The mechanism of association of the above alleles is unknown. The presentation of similar T cell epitopes by HLA-B60 and HLA-B*27:05 could suggest both an arthritogenic peptide or molecular mimicry mechanism being involved (Lopez et al., 1994). Alternatively, since other alleles such as B38 and B14 express C67, the formation of aberrant heavy chain dimers may be involved in disease pathogenesis, although C67 is not shared by B40 and B52

alleles. Hence, further studies are required to determine the mechanism of AS-associations of the above alleles.

In addition to the above MHC class I alleles, which significantly increase the susceptibility to AS in HLA-B27-negative patients, several studies have implicated an association with MHC class II loci and AS. Associations with the HLA-DRB1 alleles, particularly HLA-DRB1*01, have been reported (Brown et al., 1998) (Said-Nahal et al., 2002) (Sims et al., 2007). Other genes within the MHC class II region that have been reported to increase susceptibility to AS are Low Molecular weight Proteasome (LMP) genes which encode components of the proteasome that degrades cytoplasmic proteins and generates antigenic peptides for loading onto MHC class I molecules (Vinasco et al., 1998) (Fraile et al., 1998b) (Maksymowych et al., 2000) (Vargas-Alarcon et al., 2004).

Furthermore, several studies identified a cluster of AS associated genes within the class III MHC region including MHC-class I related chain gene A (MICA) (Singal et al., 2001), TNF- α (Gonzalez et al., 2001) (Hohler et al., 1998) and heat shock protein 70 (HSP70) gene polymorphisms (Vargas-Alarcon et al., 2002). The latter association which was found in Mexican AS patients which were both positive and negative for HLA-B27, was not replicated in Spanish and Finnish AS patients (Fraile et al., 1998a) (Westman et al., 1994). Nevertheless, most of these studies did not employ a sufficient sample size or marker density. Furthermore, these genes lie in proximity to the HLA-B27 gene, hence whether these are direct associations or reflect linkage disequilibrium with other MHC genes remains to be determined.

1.4.3.2 Non-MHC genes in AS

In the last 3 years, intensive analysis utilising genome-wide association studies has led to the identification of new genes associated with AS. Definite associations i.e. supported by genome wide significant and replicated studies, have been established for the non-MHC genes ERAP1 and IL23R, and the gene deserts at 2p15 and 21q22 whose association with AS suggests the involvement of non-coding RNA in AS pathogenesis by, as yet an unknown mechanism. In light of HLA-B27 misfolding, a mechanism of AS development involving IL-23R and ERAP1 is compelling and will be further discussed.

IL23R

Before the discovery of IL-23R genetic variants being associated with AS, previous studies strongly supported the idea that the IL-23/IL-17 axis plays an important role in AS pathogenesis. The IL-23R is involved in the inflammatory response by promoting the expansion and survival of Th17 CD4⁺ T cells (Wang et al., 2009) (McGeachy et al., 2009). The expansion of Th17 cells can trigger inflammation by secretion of IL-17 which stimulates several cell types such as macrophages, osteoblasts, fibroblasts, endothelial and epithelial cells to upregulate proinflammatory cytokines including IL-1, TNF α and IL-6 (Jovanovic et al., 1998) (Nakae et al., 2003) (Kolls and Linden, 2004) and neutrophil recruitment (Katz et al., 2001) (Ye et al., 2001a). Moreover, activated Th17 cells can also secrete TNF α and IL-6 themselves (Cua et al., 2003) (Chen et al., 2006).

IL-17 is also a key mediator of chronic inflammation in animal and human models of other SpA forms such as IBD (Yen et al., 2006) (Uhlir et al., 2006), psoriasis and autoimmune/immune-mediated inflammatory diseases including rheumatoid arthritis (RA) and multiple sclerosis (MS) (Chen et al., 2006) (Zheng et al., 2007) (Melis et al., 2010). Recent work reported elevated levels of IL-17 (Wendling et al., 2007) as well as the greater frequency of Th17 cells in the peripheral blood of AS patients (Singh et al., 2007) (Jandus et al., 2008) (Shen et al., 2009) (Ciccia et al., 2009) (Bowness et al., 2011).

The evidence from the transgenic rat model suggest that there is a possible link between HLA-B27-induced UPR and the activation of (IL23)/IL-17 axis (DeLay et al., 2009). Macrophages and cells of the colon undergoing a UPR induced by HLA-B27 misfolding appeared to be polarized to produce more IL-23 (DeLay et al., 2009) which subsequently could enhance survival of Th17 cells and stimulate IL-17 production. Alternatively, Bowness and colleagues proposed another mechanism linking the strong association of HLA-B27 and IL-23 in AS development. As mentioned before, they show that cell surface HLA-B27 heavy chain dimers can stimulate the survival and proliferation of superantigen-activated KIR3DL2⁺ expressing CD4 T cells, which in turn produce higher amounts of IL-17 (Bowness et al., 2011). Furthermore, KIR3DL2⁺ Th17 cells were found to be expanded in the synovial fluid and the peripheral blood in SpA patients with active joint disease (Bowness et al., 2011). Hence, both concepts of B27 misfolding inducing UPR and activation of IL-23/IL-17 axis as well as B27-responsive IL-17-producing T cells may contribute to the pathogenesis of

AS. Hence, the discovery of IL-23R variants supports the involvement of (IL-23)/IL-17 axis in the AS pathogenesis.

ERAP1

The Genome-wide association study by the Wellcome Trust and Anglo-Australian consortium identified numerous ERAP1 polymorphisms associated with AS. The analysis of 14,500 nonsynonymous Single-Nucleotide Polymorphisms (SNPs) revealed two new SNPs strongly associated with disease in Caucasian populations from North America and the UK. This was soon confirmed in other white and non-white populations including Chinese, Korean and Portuguese (Davidson et al., 2009) (Choi et al., 2010) (Pimentel-Santos et al., 2009). The population attributable risk for ERAP1 to AS is 26%, making it the second strongest association after HLA-B27.

As I described earlier in detail, ERAP1 has two major functions. Firstly, ERAP1 can perform the final trimming of peptide precursors to their optimal length for MHC class I antigen presentation (Saric et al., 2002) (York et al., 2002) (Chang et al., 2005) (Saveanu et al., 2005) (Hammer et al., 2006) (Hammer et al., 2007). Secondly it has been proposed to be involved in shedding cytokine receptors such as TNFR1, IL-6Ralpha, and IL-1RII (decoy IL-1 receptor) (Cui et al., 2002) (Cui et al., 2003b) (Cui et al., 2003a). None of these functions can be excluded regarding a potential role for ERAP1 in inflammatory arthritis.

The strongest associated SNPs in ERAP1 were rs30187 and rs27044 encoding K528R and Q730E variants respectively. *In vitro* enzyme studies revealed that the ERAP1 K528R mutant exhibited altered peptide processing characteristics

(Evnouchidou et al., 2011) (Kochan et al., 2011). Although, K528R is located away from the ERAP1 active site as suggested by crystallographic data, this mutation may possibly relate to impaired interdomain interactions. Most significantly these studies revealed that altered enzymatic properties of ERAP1 variants were specific to HLA-B27–restricted antigenic peptide precursors (Evnouchidou et al., 2011). Furthermore, ERAP1 AS-associated polymorphisms have been shown to correlate with the levels of expression of these genes (Dixon et al., 2007). That was recently confirmed by Campbell et al. who reported higher protein levels of ERAP1 in DC generated from HLA-B27 positive individuals (Campbell et al., 2011). Hence it is reasonable to speculate that observed altered catalytically properties of ERAP1 variants may involve mechanisms by which HLA-B27 confers susceptibility to AS. In the absence of ERAP1, a novel set of MHC class I–peptide complexes has been observed, suggesting that an altered set of peptides can be made available to bind to heavy chain. Such abnormal peptide processing could affect the redox state of HLA-B27 leading to misfolding and activation of the UPR (Turner et al., 2007) (Turner et al., 2005) (DeLay et al., 2009). Alternatively, such a peptide repertoire could lead to the expression of B27 free heavy chain at the cell surface in the form of monomer or dimers which could lead to abnormal immune interactions with KIRs and LIRs (Allen et al., 2001) (Kollnberger et al., 2007) (Kollnberger et al., 2002) (Allen and Trowsdale, 2004) (Kollnberger et al., 2004). Finally, presentation of altered peptides by HLA-B27 to T cells could potentially elicit more classical autoimmune responses (Lahesmaa et al., 1993) (Fiorillo et al., 1997). The latter might be of importance especially in light of studies by Hammer et al. who showed that ERAP1-deficient mouse cells are immunogenic

in wild type mice (Hammer et al., 2007). Moreover, one study reported an association of ERAP2 with AS. Since the only known function of ERAP2 is peptide trimming, this could further support antigenic presentation or altered peptide loading as a mechanism in which ERAP1 and possibly ERAP2 contribute to disease development.

So far there is no strong *in vivo* data supporting cytokine receptor cleavage as a mechanism by which ERAP1 polymorphisms could be implicated in AS susceptibility (Haroon et al., 2010). Nevertheless, altered receptor activity was recently suggested to potentially be involved in disease development. Notably, the association of IL1R2, which encodes a decoy receptor with high affinity for IL-1, with AS, was recently confirmed (Sims et al., 2008). Moreover, IL-6 levels are clearly elevated in AS (Gratacos et al., 1994) and IL-6 was found to promote development of Th17 lymphocytes (Wilson et al., 2007) (Layh-Schmitt and Colbert, 2008). Despite these findings, the serum cytokine or soluble receptor levels between patients with different ERAP1/ERAP2 polymorphisms and their haplotypes were similar (Haroon et al., 2010). Although the AS-associated ERAP1 and ERAP2 variants do not influence serum cytokine receptor levels in patients with AS, the ERAP1 high protein level recently detected in B27-positive individuals, may suggest a role in disease (Campbell et al., 2011). Supernatants from cells overexpressing ERAP1 showed increased levels of soluble TNFR1 (Cui et al., 2002). It would be reasonable to predict that the fewer cytokine receptors cleaved from the cell surface the lower the magnitude of the inflammatory response. Paradoxically, Haroon et al. observed that the levels of soluble (cleaved) TNFR correlated positively with the levels of inflammatory

markers (Haroon et al., 2010). Hence, further studies are required to determine whether ERAP1-mediated cytokine shedding has a role in AS development.

So far AS-associated ERAP1 polymorphism were found to predispose also to Multiple Sclerosis (MS) and psoriasis but not with Crohn's disease (Guerini et al., 2012) (Strange et al., 2010). Associations shared between various autoimmune diseases support the notion that there maybe a subset of crucial regulatory genes implicated in the pathogenesis of autoimmunity, despite the variation in both clinical outcomes and target organs across diseases.

2 Materials and Methods

2.1 Cell lines and antibodies

The C58 rat thymoma cell line, CEM and CEM.NKR cell lines were maintained in Roswell Park Memorial Institute (RPMI)-1640 medium (Gibco, Invitrogen) supplemented with 10% fetal bovine serum (FBS, Globepharm, Guildford, U.K. R10 medium) and 100U/ml penicillin/streptomycin (Sigma-Aldrich, U.K.) and L-glutamine and maintained in a 5% CO₂ 37°C incubator. C58.B27, B27.C67S, B27.C308S, B27.C325S, B27.C67S-C308S-C325S, B27.C101S-C164S, B27.C203S-C259S, B27.Q65R-I66K, B27.K70H-A71S, C58.A2, A2.V67C, A2.R65Q-K66I and A2.H68K-I69A, cells were generated by electroporation at 900µF, 180V and selected in 1 mg/ml G418 (Invitrogen, Paisley, U.K.). HeLa cells were maintained in Dulbecco's modified Eagle's medium with 10% FBS (D10). HeLa cells expressing the HLA-B27-Single Chain and HLA-B27-β2m-peptide single chain trimer construct (SCT) were maintained in D10 supplemented with 150µg/ml hygromycin (HygroGold, Invitrogen).

Monoclonal antibody HC10 recognises partially folded/unfolded HLA-B and C molecules (without β2m). The conformational specific mAbs ME1 and BB7.2 recognize folded HLA-B and A molecules respectively. HC10 was a kind gift from Prof. T. Elliott (Southampton University, U.K.). Anti-V5 (pK) antibody that binding to the V5 epitope tag (14 amino acids long C-terminal epitope: GKPIPPELLGLDST), was obtained from Serotec (Oxford, U.K.).

Table 2. The list of antibodies. Monoclonal (mAb) and polyclonal (pAb) antibodies utilised for western blotting (WB), immunoprecipitation (IP) and flow cytometry (FC) procedures in the thesis.

Antibody	Application and dilution/concentration		Antigen species	Catalogue number	Supplier
HC10 (mAb)	WB	1:5000	Mouse	N/A	Prof. T. Elliott (Southampton University, U.K.)
	IP	2 μ l			
	FC	20 μ l/ml			
ME1 (mAb)	FC	50 μ l	Mouse	N/A	Prof. T. Elliott (Southampton University, U.K.)
pK (pAb)	WB	1:1000	Mouse antisera	N/A	Serotec
pK (mAb)	WB	1:5000	Rabbit	N/A	Serotec
	IP	2 μ l			
anti-tapasin, Pasta	IP	3 μ l	Mouse	N/A	Prof. Peter Cresswell
anti-tapasin, Giles	WB	1:3000	Rabbit antisera	N/A	Prof. T. Elliott (Southampton University, U.K.)
anti-TAP1 (pAb)	IP	3 μ l	Rabbit	csa-620	Stressgen
anti-CNX (pAb)	WB	1:5000	Rabbit	SPA-865	Stressgen
	IP	5 μ l			
anti-CRT (pAb)	WB	1:5000	Rabbit	SPA-600	Stressgen
	IP	5 μ l			
anti-ERp57 (pAb)	WB	1:5000	Rabbit	ab 10287	Abcam
	IP	2 μ l			
anti-ERp57 (mAb)	WB	1:10000	Mouse	spa-725	Stressgen
anti-ERp72 (pAb)	WB	1:5000	Rabbit	SPS-720	stressgen
anti-PDI (pAb)	WB	1:5000	Rabbit	SPA-890	Stressgen
	IP	8 μ l			
anti-BiP (mAb)	WB	1:5000	Rabbit	SC-13539	Santa Cruz Biotechnology, Inc.
anti-b2m, BBM.1	WB	1:5000	Rabbit	ab18605	Abcam
GAPDH	WB	1:5000	Rabbit	N/A	Dako
HRP	WB	1:10000	goat, anti-mouse	N/A	Dako
HRP	WB	1:5000	Rabbit	N/A	Sigma
anti-CD107-PE	FC	2 μ l	Mouse	555801	BD Pharmingen
anti-CD8-APC	FC	2 μ l	Mouse	561953	BD Pharmingen
W6/32-FITC	FC	20 μ l/ml	Mouse	N/A	Prof. T. Elliott (Southampton University, U.K.)

The anti-ERp57 antisera and anti-B2m were obtained from Abcam (Abcam, Cambridge, U.K.). The anti-TAP1, anti-PDI and anti-ERp72 mAbs were obtained from AbD Stressgen (Oxford, U.K.). Anti-TAP2 sheep antisera was a kind gift from Dr. Simon Powis, St Andrews University. The anti-tapasin antibody (Pasta) was a kind gift from Prof. Peter Cresswell. Horseradish peroxidase–conjugated goat anti-mouse secondary antibody was obtained from Dako (Glostrup, Denmark) and anti-rabbit monoclonal HRP from Sigma. Fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG was obtained from Sigma.

2.2 Site-direct mutagenesis

HLA-B*27:05 and HLA-A*02:01 cDNAs were subjected to site-directed mutagenesis using the QuickChange (Stratagene, La Jolla, CA) methodology and sequenced to confirm mutagenesis. pCR3.1 (Invitrogen) containing cDNA were PCR-amplified using Pfu DNA polymerase (Promega) according to the manufacturer's protocol with complimentary sense and antisense primers carrying codons for the required substitutions. PCR conditions used were 95°C 30 secs 1x cycle, 95°C 30 secs, 55°C 1 min, 68°C 12 mins for 15 cycles. The product were then treated with DpnI (Promega) endonuclease, specific for methylated and hemimethylated DNA, hence the parental DNA template was digested. The vector DNA carrying the desired mutations was amplified in *Escherichia coli* DH5alpha competent cells. Plasmid DNA was isolated with a Plasmid Isolation Kit (Qiagen). All constructs were verified by sequencing (Dundee University, Sequencing Service, U.K.).

2.3 Transient transfection

PcR3 plasmid containing cDNA for transfection was transformed in DH5 α Escherichia coli bacteria, amplified using the mini- and maxi-prep kits (Qiagen) and purified by isopropanol (Sigma) precipitation. Plasmid DNA concentration was determined by spectrophotometry. 5×10^5 cells were plated out in 6 well plates. 100 μ l of NaCl solution (Polyplus Transfection) and 3 μ l jetPEI transfection reagent (Polyplus Transfection) was mixed with 100 μ l of NaCl containing 2 μ g of cDNA (Polyplus Transfection) and vortexed 10secs. The DNA-PEI mix was incubated at room temperature for 10 mins before adding drop wise to cells. Cells were harvested and analysed 24hrs post transfection.

2.4 Primers

The mutation forward primers (F) (see below) and respective reverse (R) complement primers used in this work were as followed:

(B27.C67S)_F 5'-CGGGAGACACAGATCAGCAAGGCCAAGGCA-3'

(B27.C67S)_R 5'-TGCCTTGGCCTTGCTGATCTGTGTCTCCCG-3'

(B27.C101S)_F 5'-CAGAATATGTATGGCTCCGACGTGGGGCCGGAC-3'

(B27.C101S)_R 5'-GTCCGGCCCCACGTCCGAGCCATACATATTCTG-3'

(B27.C164S)_F 5'-TACCTGGAGGGCGAGTGCGTGGAGTGGCTCCGC-3'

(B27.C164S)_R 5'-GCGGAGCCACTCCACGCACTCGCCCTCCAGGTA-3'

(B27.C203S)_F 5'-GAGGCCACCCTGAGGTCCTGGGCCCTGGGCTTC-3'

(B27.C203S)_R 5'-GAAGCCCAGGGCCCAGGACCTCAGGGTGGCCTC-3'

(B27.C259S)_F 5'-GAAGAGCAGAGAACATCCCATGTACAGCATGAG-3'

(B27.C259S)_R 5'-CTCATGCTGTACATGGGATGTTCTCTGCTCTTC-3'

(B27.C308S)_F 5'-GTCGCTGCTGTGATGTCTAGGAGGAAGAGCTCA-3'

(B27.C308S) _R 5'-TGAGCTCTTCCTCCTAGACATCACAGCAGCGAC-3'

(B27.C325S) _F 5'-TACTCTCAGGCTGCGTCCAGCGACAGTGCCCAG-3'

(B27.C325S) _R 5'-CTGGGCACTGTGCTGGACGCAGCCTGAGAGTA-3'

(A2.V67C) _F 5'-GGGGAGACACGGAAATGCAAGGCCCACTCA-3'

(A2.V67C) _R 5'-TGAGTGGGCCTTGCATTTCCGTGTCTCCCC-3'

(R65Q-K66I) _F 5'-GACGGGGAGACACAGATCTGCAAGGCCCACTCA-3'

(R65Q-K66I) _R 5'-TGAGTGGGCCTTGCAGATCTGTGTCTCCCCGTC-3'

(H70K-S71A) _F 5'-AAATGCAAGGCCAAGGCACAGACTCACCGA-3'

(H70K-S71A) _R 5'-TCGGTGAGTCTGTGCCTTGGCCTTGCATTT-3'

2.5 FACS analysis and Statistics

Cells were incubated with the appropriate antibody for 30 mins at 4°C, then washed 3x in PBS (phosphate-buffered saline, 1% FBS, 0.1% sodium azide-FACS buffer), followed by incubation with FITC conjugated anti-mouse IgG (Sigma, 50 µl), washed 3x and fixed with 1% formaldehyde in PBS. Staining of HLA-A2 was performed directly with FITC conjugated anti-HLA-A2 antibody (BB7.2). Analysis was performed using CellQuest software on a FACS Calibur flow cytometer (Becton Dickinson). For intracellular pK staining, cells were fixed in 3.8% paraformaldehyde (PFA)/PBS for 10 mins, followed by permeabilisation with 1x FACSPerm buffer (BD Biosciences). Permeabilised samples were incubated with anti-pK antibody (1mg/ml) overnight at 4°C, washed 3x with FACS buffer, followed by incubation for 1hr at 4°C with anti-mouse FITC conjugated antibody. Samples were washed 3x prior to acquisition on FACS Calibur.

Where appropriate, the results were analyzed by the two-way ANOVA test using GraphPad Prism software.

2.6 Quantitative Polymerase Chain Reaction

Total RNA was extracted from cells using RNeasy Mini kit (Qiagen). Briefly, 5×10^6 cells were lysed in 350 μ l of RLT buffer followed by addition of 600 μ l followed by addition of 350 μ l of RNase free 70% ethanol and mixed thoroughly. Homogenised samples were centrifugated at 8000xg for 1 min in RNeasy spin columns. Samples were washed 1x with 700 μ l RW1 buffer and then 2x with 500 μ l RPE buffer followed by RNA elution in 50 μ l RNeasy free water.

To remove genomic DNA, samples were treated with DNase enzyme (Turbo DNA-free kit, Ambion). Each RNA sample was digested with 1 μ l of DNase enzyme in the presence of 1xTURBO DNase buffer at 37°C for 30 mins. Samples were inactivated by incubation with 5 μ l of DNase Inactivation Reagent at room temp for 5 mins followed by centrifugation at 8000xg for 2mins. RNA was stored in RNase free tubes at -80°C. RNA samples were reverse transcribed into cDNA using cDNA-Synthesis Kit (Invitrogen).

Quantitative PCR was performed using probes and primers provided by Dr Helen North (University Southampton). B2705qPCR Forward primer: 5'-GACACAGATCTGCAAAGGCCA-3', B2705qPCR Reverse primer: 5'-TTGTAGTAGCGGAGCAGGGTC-3' and B2705qPCR Probe 5'-CGCAGGTCCTCTCGGTCAGTCTGTG-3' which was 5'end labelled with 6-FAM and 3' end labelled with TAMRA. The standard used was rat β -actin.

2.7 Rapid acidification and alkylation

Briefly, 7×10^5 cells were incubated at $37^\circ\text{C} \pm 5$ mM dithiothreitol (DTT, Fluka), ± 0.5 mM diamide (Sigma) for 15 mins, precipitated with ice cold 10% trichloroacetic acid (TCA, Fluka)/ phosphate buffered saline (PBS) (200 μ l) at 4°C for 10 mins, then centrifuged at 14,000rpm at 4°C for 10 mins. Pellets were washed in acetone, air-dried, and resuspended in non-reducing sample buffer with or without 4-acetamido-4'-maleimidylstibene-2,2'-disulfonic acid (AMS) (Invitrogen, San Diego, CA) at 10 mg/ml and incubated for 15 mins at 37°C .

2.8 Single-chain Trimer constructs and cell line generation

The single chain constructs were kindly provided by Dr. Keith Gould, Imperial College, London. Briefly, the single-chain trimer comprises the human $\beta 2\text{m}$ amino-terminal hydrophobic signal sequence, the peptide sequence SRYWAIRTR (residues 383–391 of influenza virus nucleoprotein peptide [NP]), a GGGGGG(SGG)₃ linker, the human $\beta 2\text{m}$ sequence, a second (GGGGS)₃ linker, and MHC class I heavy-chain sequence. Isogenic HeLa cell lines stably expressing HLA class I constructs tagged with a V5 epitope (sequence GKPIPPELLGLDST), at the C-terminus were generated by Dr. Darren Nesmith using the Invitrogen Flp-In™ system. HeLa cells were transfected with the Flp recombination target (FRT) site plasmid vector pFRT/lacZeo, and a clone containing two integrated copies of the plasmid was identified by Southern blot analysis. This clone, designated H2Z, was β -galactosidase positive and Zeocin™ resistant. HLA class I constructs for expression were cloned into the plasmid pEF5/FRT/V5-D-TOP® vector, and checked by full DNA sequencing.

The relevant FRT-containing expression plasmid was co-transfected together with the plasmid pOG44, which expresses the Flp recombinase, into H2Z cells, and cells in which homologous recombination events had occurred were selected using hygromycin B. Cells which were recombinant at both target integration sites were identified by the fact that they did not express β -galactosidase, and were ZeocinTM-sensitive thus HeLa cell lines expressing two copies of each construct were generated. HLA-B27 and HLA-B35 constructs were expressed either under the Cytomegalovirus (CMV) or Elongation Factor 1 α (EF1 α) promoters.

2.9 Cell lysis

Cells were pretreated in 20 mM N-methylmaleimide (NEM) (Sigma) in 1xPBS pH 7.0 for 15 min on ice followed by lysis in 1% Nonidet P-40 (US Biological) containing lysis buffer (150 mM NaCl, 10 mM Tris, pH 7.5) supplemented with 1x complete protease inhibitors (Roche Applied Science), 1mM phenylmethylsulfonyl fluoride (PMSF) (Sigma) and 10 mM NEM. Lysates were analyzed under reducing or nonreducing conditions on 8% SDS-PAGE gels, followed by immunoblotting. Where necessary, samples were reduced by incubation with 100mM DTT for 5 min. at 95°C.

2.10 Immunoprecipitation

Approx. 3×10^7 cells were pretreated with 20mM NEM on ice for 15 min, then resuspended in lysis buffer (150mM NaCl, 10mM Tris, pH 7.5, 1mM phenylmethylsulfonyl fluoride, 10mM NEM) containing 1% digitonin (WAKO) or 1% NP40. Lysates were precleared with 50 μ l CL4B Sepharose (Sigma), 20 μ l

Protein A (Sigma) and/or 20µl Protein G (Sigma) sepharose beads for 30 mins at 4°C, followed by immunoprecipitation with appropriate antibody for 2hrs at 4°C. Immunoprecipitates were washed 5x 0.5mls with 1% NP40 buffer and resuspended in non-reducing sample buffer.

2.11 Immunoblotting

Samples were resolved by 8% SDS-PAGE (Resolving gel: 8% Acrylamide (Sigma-Aldrich), 0.375M Tris pH 8.8, 0.1% SDS, 0.1% Ammonium persulfate (APS) and 0.04% N,N,N',N'-Tetramethylethylenediamine (TEMED). Stacking Gel: 5% Acrylamide, 0.125 M Tris pH 6.8, 0.1% SDS, 0.075% APS and 0.075% TEMED) for ~120 min at 100V in 1x Tris-Glycine SDS running buffer (25mM Tris, 192mM glycine and 0.1% w/v SDS pH8.3) in order to resolve all proteins. The prestained protein ladder PageRuler (Fermentas, York, UK) was run along the samples to determine the proteins' size.

Gels were then wet transferred in 1x transfer buffer (25mM Tris, 192mM glycine) for 1 h at 100V onto nitrocellulose (BA85, Whatmann, UK). Membranes were then blocked in 5% (w/v) skimmed milk powder (Tesco) in PBST (0.1% Tween 20 (Sigma-Aldrich)/PBS) either 2 x 30 min in room temp or overnight at 4°C to block non-specific binding sites. Subsequently the membranes were incubated with appropriate primary antibodies (see Table 2) diluted in PBST (0.1% Tween 20 (Sigma-Aldrich)/PBS), either overnight at 4°C or a minimum of 1hr at room temperature. The membranes were washed 2 x 15 min in room temp in washing buffer (PBST) before adding the secondary antibody. The membranes incubation with a HRP-conjugated secondary antibody (diluted in

0.1% Tween/PBS) was performed for 1 hour at room temp. Afterwards, the membranes were washed 3 x 15 min in 0.1% Tween/PBS.

Images were developed by chemiluminescence using SuperSignal Femto (Pierce, Illinois, UK) or ECL reagent (GE Healthcare) according to manufacturer's instructions. Dried membranes were wrapped in cling film and chemiluminescence was measured by autoradiography using ECL Hyperfilm (GE Healthcare) developed in a Developer Xograph imaging system CompactX4.

If appropriate, to re-probe the membrane, the primary and secondary antibodies were stripped off by incubating the membrane in stripping buffer at 60°C for 45 min. Subsequently, the membranes were washed in washing buffer, blocked and re-incubated with appropriate different antibodies.

Endo H digestion

Non-reduced cell lysates were digested with 1µl of Endoglycosidase H (Roche) for 1hr at 37°C, followed by separation on SDS-PAGE and immunoblotting.

2.12 Pulse-Chase analysis

Approximately 5×10^6 cells were preincubated with methionine/cysteine free RPMI (Gibco), for 20 mins, labelled with 7.2 Mbq ^{35}S -Trans label (MP Biologicals) for 10 mins, washed and resuspended in R10 media. Aliquots were removed at specific time points indicated, lysed in 1% NP40 lysis buffer supplemented as described above. Samples were precleared with sepharose 4B/protein A sepharose (Sigma) for 30 mins at 4°C, and immunoprecipitated

with pK conjugated to 20µl protein A sepharose for 1 hr at 4°C. Immunoprecipitates were washed 3x 0.5mls with 1% NP40 buffer and resuspended in non-reducing sample buffer. Non-reduced samples were digested with 1µl of Endoglycosidase H (Roche) for 1hr at 37°C, followed by separation on 8% SDS-PAGE. Resolved gel slices were dried for 2h at 80°C and then were subject to autoradiography using Imaging Screen K (Kodak) storage phosphor screens in a light tight cassette for up to 9 weeks. Film was developed in a Developer Xograph imaging system CompactX4 and radio-emission was analysed using TotalLab-Quant software (TotalLab LTD.)

2.13 Two dimensional electrophoresis

Two-dimensional electrophoresis was performed according to manufacturer's conditions with Bio-Rad Mini-Protean® equipment. The cell lysates for 2D-electrophoresis were resolved in the 2D sample buffer containing 9.5M UREA, 2% NP40, 1.6% of 6-8 and 0.4 of 3-10 Amylocyte. The samples were resolved in the first dimension tube gels containing 9.2M UREA, 4% Acrylamide, 2.0% NP40, 0.4% 3-10 Ampholyte (Sigma), 0.01% APS and 0.1% TEMED. Samples were run at 500V for ~4 hrs in 20mM NaOH upper chamber buffer and 10mM H₃PO₄ lower chamber buffer. The second dimension gel was prepared and resolved on a 8% SDS-PAGE gel described above (see Immunoblotting)

2.14 Cytotoxic T cells-killing assay

HLA-B27 specific influenza NP T cells were plated in 96 well plates (5 x 10⁴/well) and stained with anti-CD107a Ab directly conjugated to Phycoerythrin (PE), by incubation for 1hr on ice (2µl/well). HeLa cells were pulsed with NP-

peptide (100µl) and incubated 1hr, 37°C, then washed 3x in PBS. Peptide pulsed or un-pulsed HeLa cells were added to the T cells at a ratio 1:1 of effector to target cells. After 1hr incubation at 37°C, Brefeldin A (1 µl/ml) was added and left for 3 hrs at 37°C in 5% CO₂ incubator. The pelleted cells were stained with anti-CD8 directly conjugated to allophycocyanin (APC) (2µl/well) for 0.5hrs on ice then washed 3x in FACS buffer. Samples were acquired on the Cyan ADP flow cytometer and analysed by flow cytometry software Flowjo.

3 Results

3.1 Role of unpaired and structurally conserved cysteines in HLA-B27 dimer formation

3.1.1 Introduction and Aims

HLA-B27 is strongly associated with a group of inflammatory arthritic disorders known as the Spondyloarthropathies (SpAs) (Brewerton et al., 1973). The unusual biochemistry of HLA-B27 has been proposed to participate in disease development, especially the enhanced ability of HLA-B27 to form disulphide linked heavy chain dimers (Allen et al., 1999) (Turner et al., 2005) (Dangoria et al., 2002) (Bird et al., 2003). The mechanism of dimerisation however remains elusive. All MHC class I heavy chains possess four conserved cysteine (C) residues, which form two structurally important disulphide bonds within the α 2-domain between C101-C164 and the α 3-domain between C203-C259. In addition, HLA-B27 expresses three unpaired cysteines at position (p) 67, p308 and p325 expressed in the α 1 domain, boundary of the transmembrane and cytoplasmic domain and within the cytoplasmic tail respectively. Nevertheless, their role in B27 biology and potential participation in B27 dimer formation has not been fully elucidated.

So far most of the attention in the dimerisation process has been dedicated to C67, located within the α -helix of the α 1 domain, which was found to be important in the formation of both recombinant and cell surface heavy chain-

dimers (Allen et al., 1999) (Bird et al., 2003). Allen et. al. were the first who described a possible role for C67 in HLA-B27 homodimer formation, since purified HLA-B27 refolded *in vitro* without β 2m had a tendency to form disulphide-linked dimers through the unpaired C67 residue (Allen et al., 1999). However, several reports suggest that B27 dimer formation is not exclusively dependent on the unpaired C67 (Antoniou et al., 2004) (Tran et al., 2004). Introduction of C67 into the non-AS-associated MHC class I allele HLA-A2 failed to induce dimerisation (Antoniou et al., 2004). Accordingly, replacement of C67 to serine does not abrogate disulphide linked homodimers of B27 when detected in transgenic rats, which continue to develop inflammatory disease (Tran et al., 2004). The presence of disulphide bonded dimers in the absence of C67 and their apparent absence when C67 is introduced into non-HLA-B27 heavy chain alleles, suggests that either the unpaired C308 and/or C325 or the conserved cysteines at p101, p164, p203 and p259 might participate in HLA-B27 dimer formation. Furthermore, the C67 residue is shared by almost all HLA-B27 subtypes (except B*2718 and B*2723), including those which exhibit no or weak associations with AS (B*27:06 and B*27:09). Finally, although relatively rare, C67 is also present within the sequence of non B27 alleles (HLA-B14, -15, -38, -39 and -73) (Kostyu et al., 1997) (Bird et al., 2003) whereas other HLA alleles that lack C67 can also form heavy chain dimers (Dangoria et al., 2002) (Nossner and Parham, 1995).

Indeed, it seems that C67 participates mainly in the formation of cell surface homodimers (Bird et al., 2003). Using cell surface radio-labelling with iodine (I)¹²⁵, cell surface heavy chain dimers were shown to arise from the recycling of

fully folded HLA-B27 molecules through the endocytic pathway (Bird et al., 2003). Notably, they seem not to be related to ER resident dimers since this population was shown to be constitutively sensitive to Endo H treatment which indicates an immature phenotype and thus a lack of trafficking from the ER to the Golgi apparatus (Dangoria et al., 2002) (Bird et al., 2003). These ER resident dimers appear to be the product of the slow HLA-B27 maturation kinetics and its tendency to misfold (Antoniou et al., 2004) (Mear et al., 1999). The former was demonstrated in a study wherein temperature-induced slowing of the assembly rate of the non-disease associated allele HLA-A2 elicited its dimerisation (Antoniou et al., 2004). HLA-B27 was demonstrated by Mear et al. to exhibit an enhanced susceptibility to undergo ER-associated degradation (ERAD), which is a cellular pathway that disposes of misfolded proteins within the ER (Hughes et al., 1997). It was therefore concluded that the HLA-B27 heavy chain exhibited an enhanced tendency to misfold (Colbert, 2000b) (Colbert, 2000a) (Mear et al., 1999).

In addition to different assembly mechanisms of cell surface versus ER resident dimers, the latter were proposed to also engage the conserved structural cysteines (Antoniou et al., 2004). In our laboratory it was previously demonstrated that substitution of C164 abrogated dimer formation within the ER (Antoniou et al., 2004). The rationale for the role of conserved cysteine residues in aberrant interchain disulphide bonding was supported by the observation that during MHC class I assembly, C101 and C164 seem to remain in a reduced state until appropriate peptide binding occurs (Dick et al., 2002). Hence, this could hypothetically allow or enhance the possibility for intermolecular

disulphide bonding during the prolonged folding of HLA-B27 within the ER. On the other hand it does not exclude the possibility that by mutating C164, folding changes could be induced that prevented C67-C67 interactions. Taken together, the above studies suggest that HLA-B27 dimerisation is not solely due to C67-C67 interactions.

The potential involvement of other unpaired cysteine residues at p325 and 308 in HLA-B27 dimerisation, can not be fully excluded. Direct involvement of C325 in HLA-B27 homodimer formation has recently been reported but in a novel context (Lynch et al., 2009). Disulphide linked dimers composed of fully folded MHC class I molecules have been detected within exosomes. These exosomes are small vesicles formed by invagination of endosomal cell membrane to generate multivesicular bodies which can fuse with the plasma membrane and release these internal vesicles (exosomes) into the extracellular space (Raposo et al., 1996) (Johnstone et al., 1987). Exosomes are secreted by a wide range of mammalian cell types and are found in body fluids including blood, urine, ascites and amniotic fluid (Johnstone et al., 1989) (Raposo et al., 1996) (Heijnen et al., 1999) (Zitvogel et al., 2000) (van Niel et al., 2001) (Clayton et al., 2005). Secreted exosomes are biologically active entities which seem to play a role in tumour biology and immune regulation (Wolfers et al., 2001) (Skokos et al., 2002) (Clayton and Tabi, 2005). The exosomal-derived dimers were not only composed of two HLA-B27 molecules but were also detected as heterodimers containing HLA-B27 disulphide bonded to HLA-A2 (Lynch et al., 2009). However, the exosomal environment favours MHC class I homodimerisation due to low levels of reduced glutathione (GSH) (Lynch et al.,

2009), which is a thiol-containing compound which participates in maintaining the reducing environment within the cell (Bass et al., 2004) (Jessop and Bulleid, 2004). The exosomal environment is possibly parallel to the oxidising environment of the ER lumen where the ratio of the reduced glutathione (GSH) to the oxidised glutathione (GSSG) is 1:1 to 3:1 which favours disulphide bond formation, in contrast to the cytosol reducing environment, where the GSH:GSSG ratio is 30:1 to 100:1 (Hwang et al., 1992) (Bass et al., 2004). Nevertheless, despite the enhanced reducing cytosolic potential, there are some circumstances that can lead to disulphide bonding even within the cell cytoplasm. It has been shown that during oxidative stress, which is thought to be a downstream effect of protein misfolding (Kadowaki et al., 2005) (Zhu et al., 2007), thiols of cytoplasmic cysteines are vulnerable to oxidation by reactive oxygen species (ROS) (Cumming et al., 2004) (Jakob et al., 1999). Particularly, hydrogen peroxide (H_2O_2), a relatively mild oxidant implicated in oxidative stress, can oxidize cysteine sulfhydryl (-SH) groups to a cysteine sulfenic acid (Cys-SOH) or disulphide bonds (Thomas and Mallis, 2001).

As well as HLA-B27, most HLA-B alleles express a cytosolically located cysteine at p308. This residue, located on the border of the stop transfer sequence, has also been found to be capable of forming disulphide bonds (Chambers et al., 2008). A complex of HLA-B35 disulphide bonded with tapasin via C308 of the heavy chain and possibly C420 of tapasin, have been detected in an in vitro protein translation system (Chambers et al., 2008).

Both C308 and C325 residues have also been reported to participate in other aspects of MHC class I biology. Mutating C308 and C325 to tryptophan and serine respectively, was shown to impair the egress of HLA-B7 from the ER and prevent MHC class I recognition by the leukocyte Ig-like 1 inhibitory receptor (LILR1) (Gruda et al., 2007). The same study reported that C308 might influence the folding of HLA-B7 by acting as a palmitoylation site (Gruda et al., 2007). Palmitoylation is a posttranslational modification involving the covalent attachment of fatty acids to cysteine residues through a thioester linkage (Bijlmakers and Marsh, 2003). Palmitoylation has been demonstrated to participate in protein trafficking between membrane compartments, endocytosis, recycling and protein stability (Blanpain et al., 2001) (Ochsenbauer-Jambor et al., 2001).

Furthermore, HLA-B27 dimers are a heterogeneous population. Engagement of different cysteine residues during the formation of aberrant intra- and intermolecular disulphide bonds and possibly occurring in different combinations might at least partially explain the detection of several different forms of HLA-B27 heavy chain dimers. Such dimeric species can be detected by different monoclonal antibodies. The conformationally specific antibody W6/32, which detects folded complexes containing peptide and β 2m (Barnstable et al., 1978), can recognise a single dimeric population (~90kD) thought to constitute heavy chains in a “folded” state (Dangoria et al., 2002) whilst HC10 can immunoprecipitate a range of dimer species between 80-100kD referred to as “unfolded” dimers (Dangoria et al., 2002) (Bird et al., 2003) (Tran et al., 2004) (Antoniou et al., 2004). The antibody, 5H7, which recognises an epitope in the

$\alpha 3$ domain of the MHC class I heavy chain, does not bind to HC10-specific dimers (Dangoria et al., 2002). These observations suggest that either HC10-reactive dimers have a disrupted $\alpha 3$ domain or the epitope is perhaps blocked by other protein(s) interacting with the heavy chain. However, the basis for the differences in HLA-B27 heavy chain dimer populations remains undetermined.

Aims

Our group and others have demonstrated that HLA-B27 dimers do not exist as a homogenous population. I therefore wanted to;

- i) determine whether these disulphide bonded populations involve unpaired cysteines other than C67 i.e. C308 and/or C325,
- ii) determine the contribution of C164 in HLA-B27 aggregation i.e. whether it is involved in a direct C164-C164 disulphide bond or participates/influences other cysteines interactions,
- iii) determine the basis for the difference in heavy chain dimer populations.

3.1.2 Results

3.1.2.1 Cysteine residues at p67, p308 and p325 influence the cell surface expression of HLA-B27

All MHC class I molecules express 4 conserved structural cysteine residues which form disulphide bonds within the $\alpha 2$ domain between C101-C164 and $\alpha 3$ domain between C203-C259 (Fig. 3.1A). In addition, HLA-B27 expresses three additional cysteines at p67, p308 and p325 (Fig. 3.1A). To determine the role of these additional unpaired cysteines in HLA-B*27:05 dimerisation, using a PCR based site directed mutagenesis protocol, each cysteine was mutated to serine, either individually and in combinations as shown in Figure 3.1B. Using electroporation followed by single cell cloning, the given mutants were stably expressed in the C58 rat thymoma cell line. The C58 rat thymoma cell line was used since the rat model for AS is the best characterized regarding disease pathology (Taurog et al., 1999).

To determine whether the introduced mutations affected cell surface expression of HLA-B27, each was analysed by FACS, using the conformationally dependent ME1 antibody which recognises heavy chain/ $\beta 2m$ /peptide complexes of HLA-B27, -B7, and -B22 heavy chains (Ellis et al., 1982), and the HC10 antibody that recognise $\beta 2m$ -free (unfolded) B and C alleles (Stam et al., 1986) (Neisig et al., 1996). Flow cytometric analysis (Figure 3.2A) revealed that fully assembled ME-1-reactive HLA-B27.C67S, C325S, C308S and C67S-C308S-C325S molecules were expressed within three-four fold less efficiently than the wild type (wt) HLA-B27 molecule at the cell surface (Figure 3.2A),

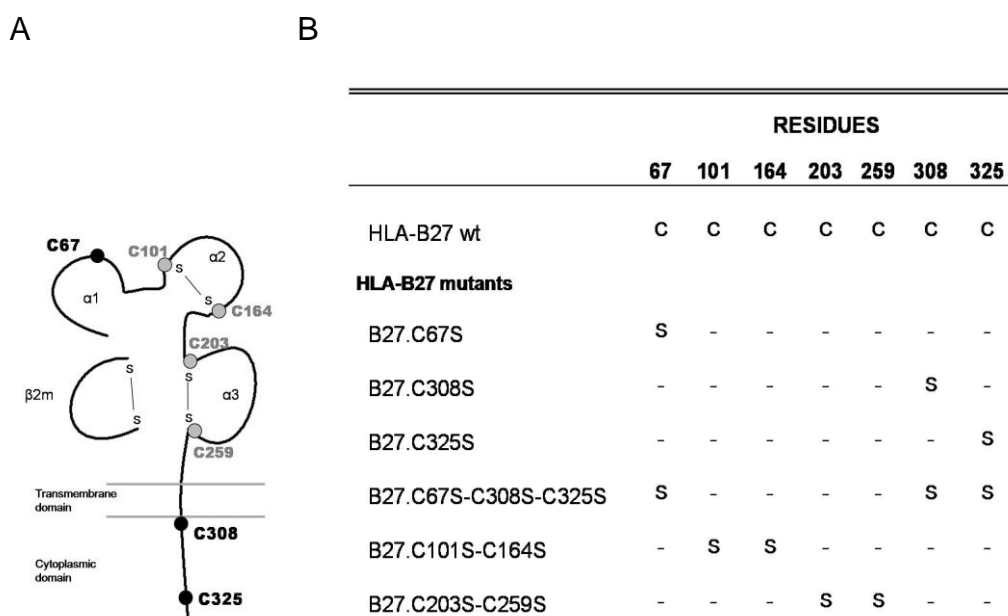


Figure 3.1. HLA-B27 and cysteine residue mutants used in this study. (A) Outline structure of HLA-B27 with highlighted conserved cysteine (C)101 and C164 within the $\alpha 2$ domain, C203 and C259 within the $\alpha 3$ domain and the unpaired C67, C308 and C325 residues. (B) HLA-B27 generated mutants contain substitution of conserved and unique C residues to serine (S). HLA-B*27:05 cDNA was subjected to site-directed mutagenesis using the QuickChange protocol.

whereas expression of B27.C308S mutant was only slightly reduced. However, cell surface expression of HC10-reactive B27 heavy chain did not differ between HLA-B27wt and the B27 cysteine mutants. Lower expression of ME1-reactive heavy chain indicated that either the B27 cysteine mutants do not attain a fully folded state and thus reside within the ER or heavy chain/ β 2m/peptide complexes have an enhance tendency to decay at the cell surface. According to the latter assumption the decrease in the folded ME1-reactive heavy chain population at the cell surface should be accompanied by the increase in the presence of HC10-sensitive β 2m-free heavy chain. However, since the HC10-reactive population is comparable between each cysteine mutant, it suggests that the B27 cysteine mutants remain within the ER.

To discount whether differences in cell surface expression of cysteine residue mutants was due to differential gene and/or protein expression levels, the HLA-B27 cysteine mutants were subjected to Quantitative Polymerase Chain Reaction (Q-PCR) and Western Blotting, respectively. With the help and supervision of Dr Nina Witt, the Q-PCR was performed using probes and primers provided by Dr Helen North (Colindale Blood Service, London) which were previously verified for their HLA-B27 specificity. HLA-B27 expression was normalised to β -actin. These analyses revealed that both HLA-B27 wt and the respective cysteine mutants expressed comparable cDNA levels (Figure 3.2B).

In order to determine protein expression levels, equal cell numbers (3×10^5) of each C58 cell line expressing the HLA-B27 cysteine mutants were harvested and lysed in 1% NP40 detergent in the presence of protease inhibitor cocktails.

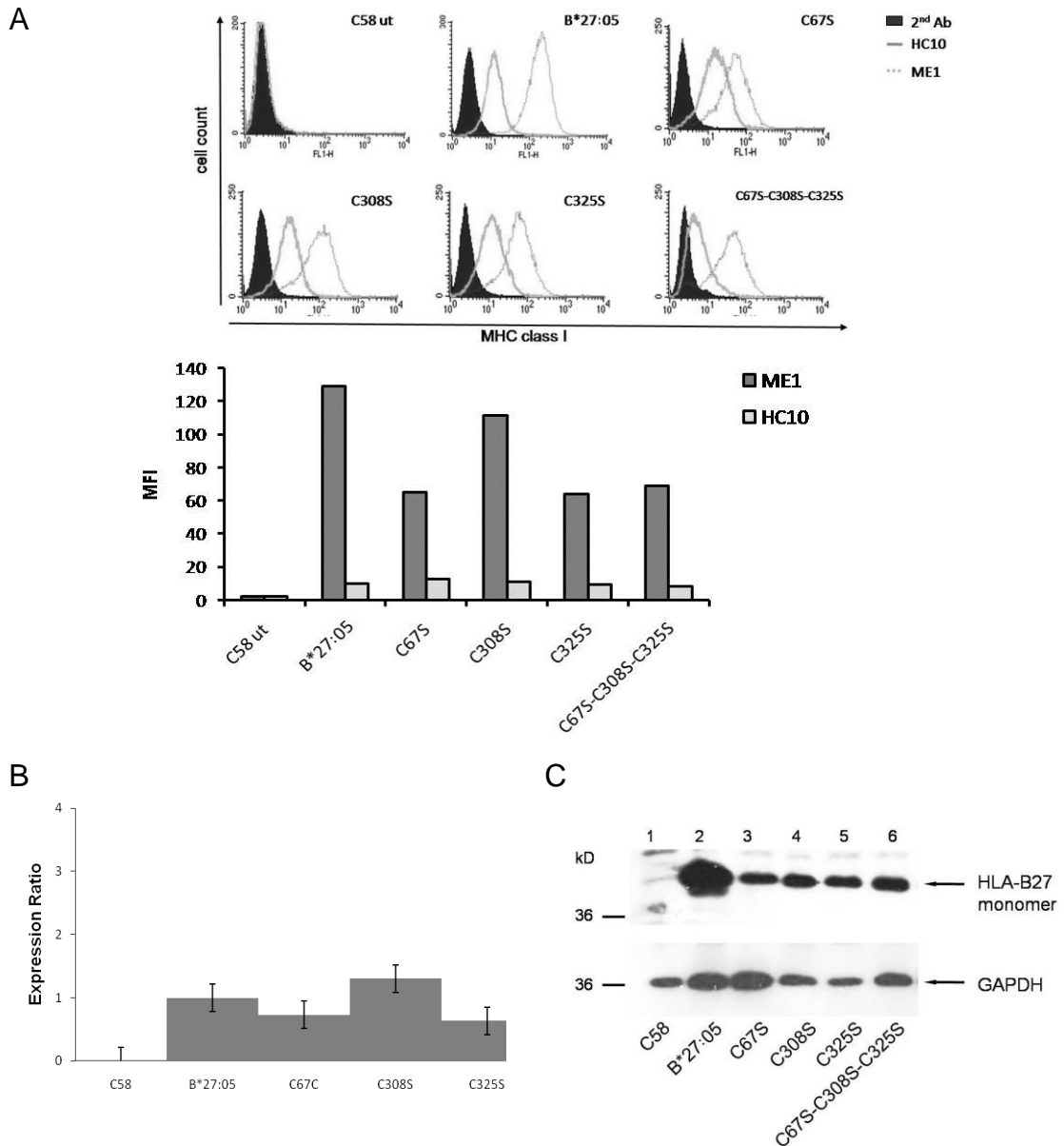


Figure 3.2. Characterisation of C58 rat thymoma cell lines expressing HLA-B27 unpaired-cysteine mutants (A) C58 cells untransfected (ut) and C58 cells expressing HLA-B*27:05 (wild type), C67S, C308S, C325S and C67S-C308S-C325S mutants were analysed for cell surface expression by FACS using HC10 and ME1 antibodies. The representative histogram plots are shown. The bar charts show the Mean Fluorescence Intensities (MFI) of the average data from five different experiments. **(B)** Equal cell numbers of C58 cell lines from B were analysed by Quantitative-PCR using B27 specific primers and normalised against β -actin. **(C)** Cell lines expressing given mutants were lysed in 1% NP40 detergent. Reduced lysates were resolved on 8% SDS-PAGE followed by immunoblotting with anti-MHC class I antibody HC10 and an antibody against the housekeeping protein, Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) for reference.

Reduced lysates were separated by SDS-PAGE, followed by immunoblotting for MHC class I heavy chain with the HC10 antibody (Figure 3.2C, top panel). The blot was then stripped and probed for Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), which is a constitutively expressed housekeeping protein to determine protein loading (Figure 3.2C, bottom panel).

Immunoblotting with HC10 revealed that HLA-B27wt is expressed at higher protein levels than the cysteine mutants as assessed by immunoblotting for house-keeping protein GAPDH. However, there appears to be no significant differences in protein levels between HLA-B27.C67S, C308S and C325S mutants (Figure 3.2C, lanes 3-6). These results suggest that the observed lower cell surface expression of the unpaired cysteine mutants might be due to the lower protein level, however, it does not exclude whether these differences are also accompanied by assembly/folding defects.

It was possible that the introduced cysteine-to-serine mutations resulted in structural perturbations/defects of the HLA-B27 heavy chain, which could further reduce their cell surface expression. As previously shown, decreasing the incubation temperature of MHC class I-transfected cells stabilizes HLA class I molecules which are suboptimally loaded with peptides, allowing their egress from the ER and transport to the cell surface (Ljunggren et al., 1990) (Zernich et al., 2004). If the mutations resulted in structural defects, incubation at the lower temperature would fail to improve or restore their cell surface expression levels to those of HLA-B27wt. Hence, to determine whether the reduced cell surface expression was either due to folding or structural defects, cells expressing each mutant were incubated at 26°C or 37°C for 16 hrs prior to FACS analysis

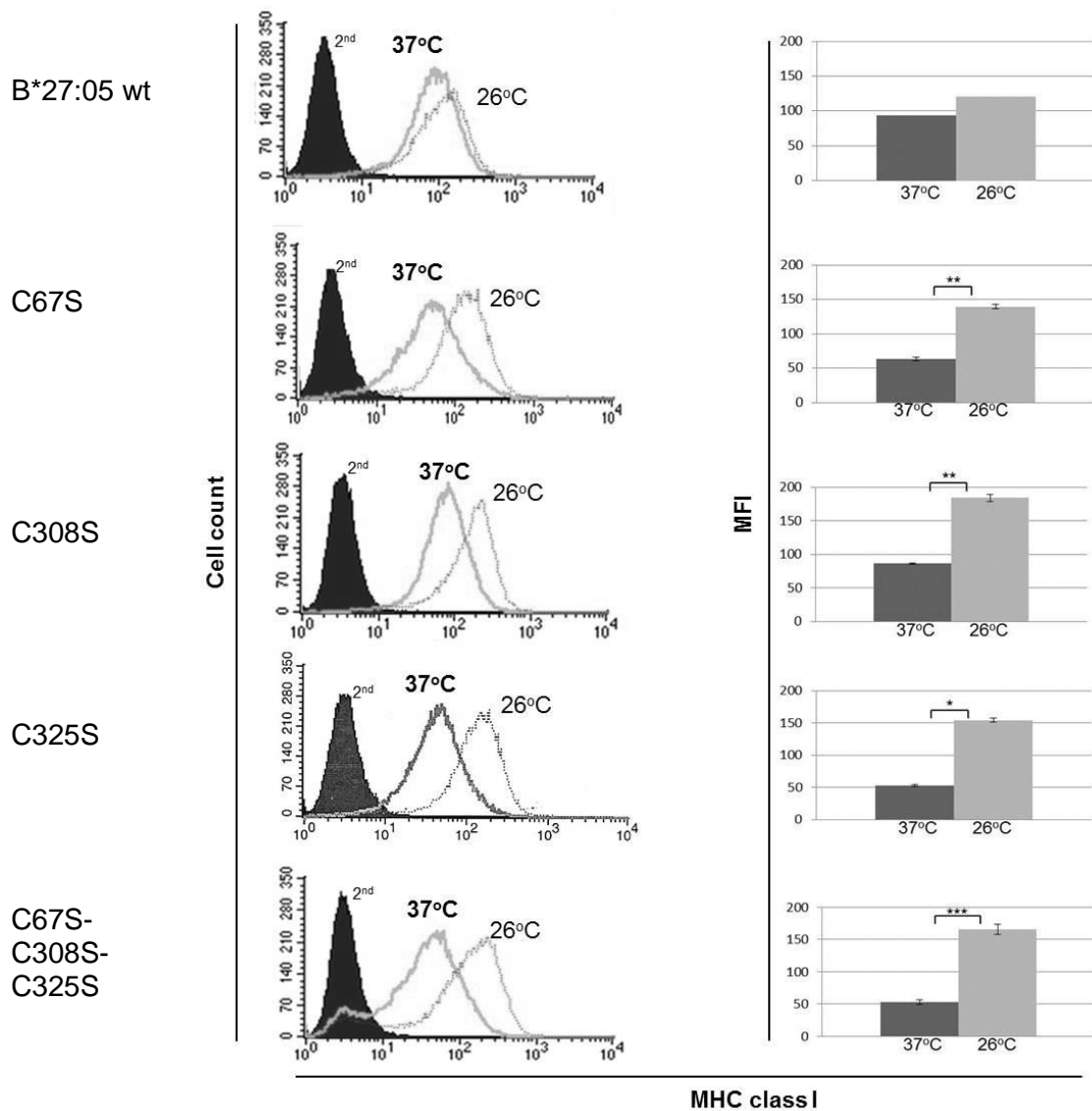


Figure 3.3. Unpaired HLA-B27 cysteine residues influence heavy chain folding. C58 cells expressing B*27:05 wild type and C67S, C308S, C325S, C67S-C308S-C325S mutants were incubated overnight at 26°C or 37°C, followed by FACS analysis with ME1. Mean Fluorescence Intensities (MFI) (right) demonstrate two to three-fold increase in cell surface expression of the unpaired cysteine mutants following incubation at 26 °C. Except results for B*27:05 wt, data are representative of 3 independent experiments. Statistical analysis was performed using the two-way ANOVA test. *P<0.001, **P<0.003, ***P<0.006.

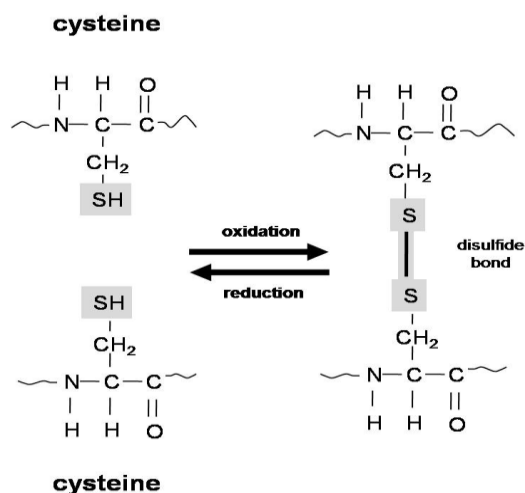
(Figure 3.3). Decreasing incubation temperature resulted in a two to three-fold increase in the cell surface expression of fully assembled (ME-1 reactive) B27-cysteine mutants (Figure 3.3). These observations suggest that residues 67, 308 and 325 influence some parameter required for efficient HLA-B27 cell surface expression and that cysteine-to-serine mutations do not cause defects in heavy chain structure.

3.1.2.2 Role of unique cysteine residues in dimer formation

One of the unusual features regarding HLA-B27 folding is its enhanced ability to form heavy chain dimers which occurs through disulphide bonding via the thiol groups of two cysteines (Figure 3.4A).

To assess the extent of *in vivo* dimerisation of HLA-B*27:05 and to avoid post-lysis oxidation events, which can potentially enhance disulphide bonding and therefore affect the detectable levels of B27 dimers, I pretreated C58.B*27:05 expressing cells with the cell permeable alkylating agent N-ethylmaleimide (NEM). NEM is a small compound that forms stable, covalent thioether bonds with sulfhydryls (e.g., reduced cysteines), enabling them to be permanently blocked to prevent further disulphide bond formation (Figure 3.4B). Alkylation of free thiols prevents any further oxidative reactions, thus preserving transient disulphide bond formation between proteins and any further post-lytic oxidation events. Following alkylation, cells lysates were separated by 8% non-reducing SDS-PAGE and immunoblotted for MHC class I heavy chain. Immunoblotting with the HC10 antibody confirmed that HLA-B27 can form high molecular weight (Mw) homodimers within the 80-110 kD size range (Figure 3.5A, left panel),

A



B

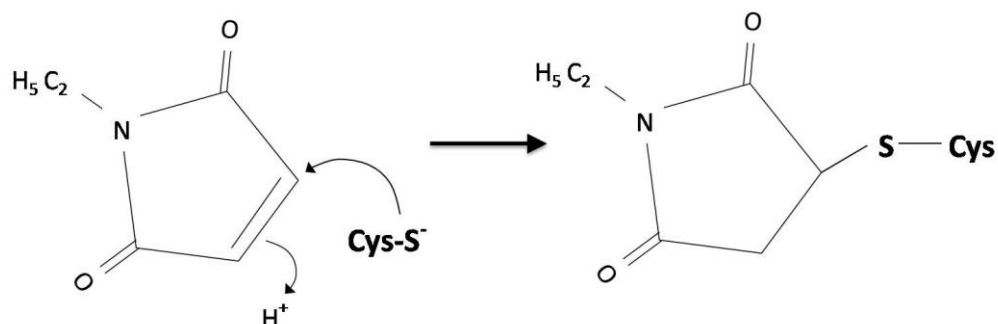
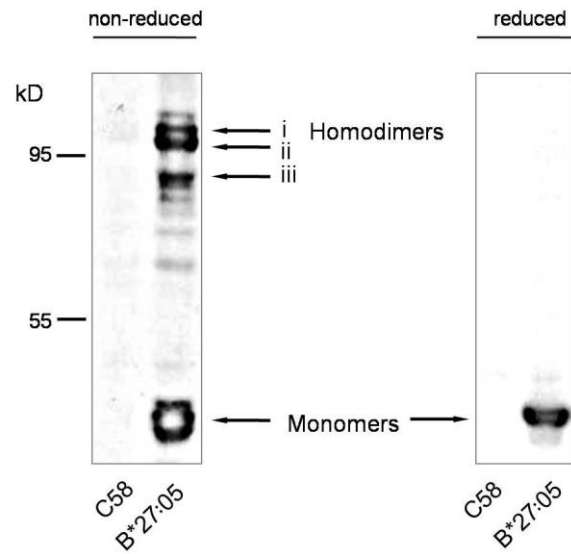
***N*-Ethylmaleimide**

Figure 3.4. Disulphide bond formation and cysteines trapping. (A) Depiction of disulphide bond formation between thiol groups (-SH) of two cysteines. (B) *N*-Ethylmaleimide (NEM) is a cell permeable alkylating agent which via its maleimide group can form stable, covalent thioether bonds with sulfhydryls (e.g., reduced cysteines), enabling them to be permanently blocked and prevent disulphide bond formation.

A



B

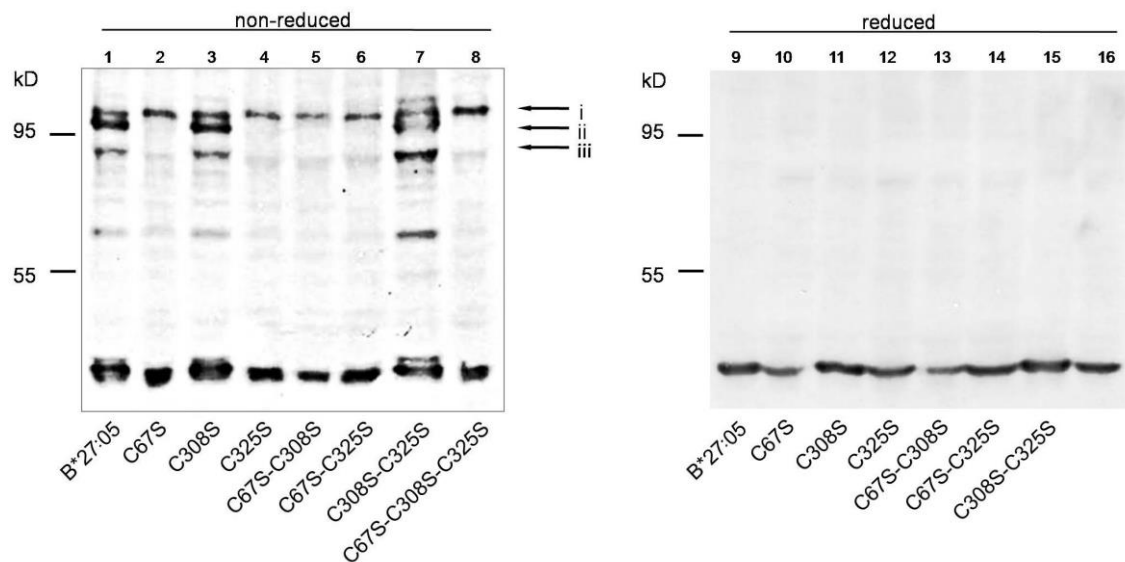


Figure 3.5. HLA-B27 unpaired cysteine residues influence homodimer formation.

(A) C58 cells expressing HLA-B*27:05 wild type and (B) B27 cysteine mutants were treated with the alkylating agent NEM prior to lysis in 1% NP40 and analyzed non-reduced (left) and reduced (right) by immunoblotting with HC10. Arrows i-iii indicate detected MHC class I high Mw species.

which disappear upon reduction with 100mM dithiothreitol (dTT) resulting in a single monomeric band (Figure 3.5A, right panel). The presence of different HLA-B27 dimers populations (Figure 3.5A, arrows i-iii) confirms our and other groups earlier studies (Dangoria et al., 2002) (Bird et al., 2003) (Tran et al., 2004) (Antoniou et al., 2004) (Figure 3.5A, right panel). Furthermore, as reduction lead to the detection of a single band, this suggested it was unlikely that these dimers populations were a result of different glycosylation states of the heavy chains.

The unpaired C67 has been previously reported to play a role in B27 homodimer formation (Allen et al., 1999). To evaluate the influence of other non-structural cysteine residues on B27 dimer formation, C58 cells expressing HLA-B27 with cysteine-to-serine substitutions at p67, p308 and p325 were subjected to NEM treatment followed by lysis and immunoblotting with HC10 antibody as described above. It seems that at least two of the B27-dimer populations can be influenced by unpaired cysteine residues (Figure 3.5B, arrows ii and iii). The dimer species indicated by arrow ii is mainly affected by the absence of C67 and C325 (Figure 3.5B, lanes 2, 4, 5, 6 and 8). Nevertheless, regardless whether C67, C308 or C325 were substituted individually (Figure 3.5B, lanes 2, 3 and 4), in paired combinations (Figure 3.5B, lanes 5, 6, and 7) or when all three have been mutated (Figure 3.5B, lane 9), HLA-B27 can still retain the ability to dimerise (Figure 3.5B, arrows i-iii, left panel). Taken together, the above experiment suggests the involvement of either C67 and/or C325 in dimer formation and indicates that also conserved cysteine residues are involved in this process.

3.1.2.3 Role of structural cysteine residues in dimer formation

Since the above results indicate that unpaired cysteine residues are not crucial for dimer formation this directed my attention to the conserved cysteine residues expressed at p101, c164, p203 and p259.

Our group has previously postulated that the conserved C164, is also involved in the formation of HLA-B27 ER resident dimers (Antoniou et al., 2004). By mutating C164, B27 demonstrated a reduced tendency to dimerise (Antoniou et al., 2004). Therefore, to address this possibility further, using an V5 C-terminally tagged HLA-B*27:05 cDNA, both the conserved $\alpha 2$ (C101S-C164S) and $\alpha 3$ (C203S-C259S) domain cysteines were mutated to serines. The steps of analysing the contribution of conserved cysteine residues in B27 dimer formation are schematically illustrated in a Figure 3.7. Firstly, to determine the role of the conserved cysteines in HLA-B27 dimer formation, HLA-B27.C101S-C164S and HLA-B27.C203S-C259S expressing C58 cell lines were pretreated with NEM to trap and maintain any transient disulphide bonded species. Lysates were generated using 1% NP40 detergent and separated by non-reducing and reducing SDS-PAGE, followed by immunoblotting with the anti-V5 epitope tag antibody pK. Immunoblotting revealed that heavy chain-dimers were ablated in both C164S and C101S-C164S mutant (Figure 3.6A, left panel). Therefore, despite C67 and the unpaired C308 and C325 being intact, these residues were not sufficient to mediate heavy chain dimerisation. The C203S-C259S mutant retained the ability to dimerise (Figure 3.6A, left panel), indicating that the $\alpha 3$ -domain cysteines do not indeed participate in dimerisation. The single dimer band evident in the HLA-B27.C203S-C259S

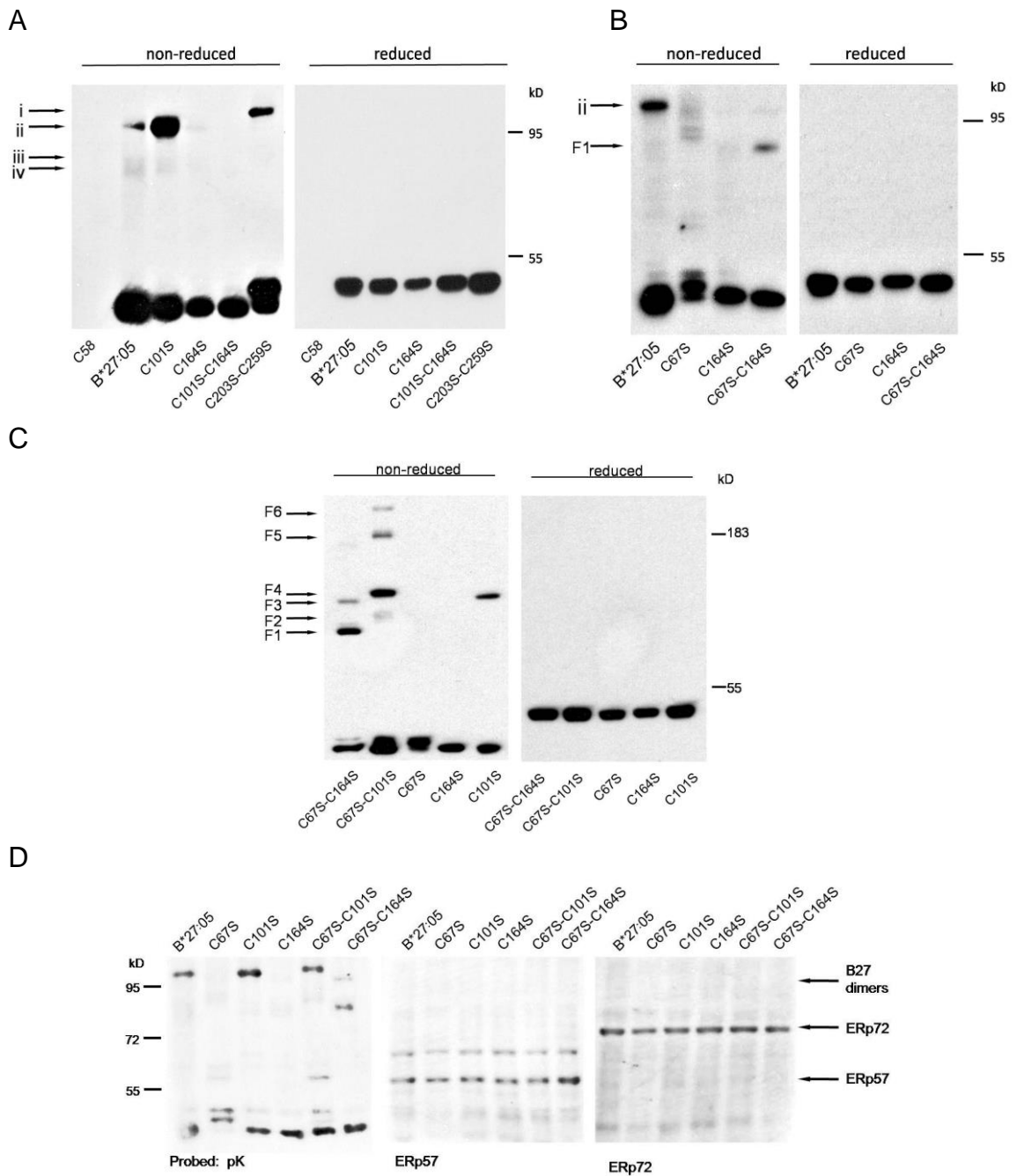


Figure 3.6. The structurally conserved cysteines C101 and C164 participate in HLA-B27 heavy chain dimer formation and interact with cysteine 67. C58 cells expressing (A) B27, C101S, C164S, C101S-C164S and C203S-C259S, (B) C67S, C164S and C67S-C164S and (C) C67S-C164S, C67S-C101S, C67S, C164S and C101S were NEM treated prior to lysis. Lysates were analysed by non-reducing (left panel) and reducing SDS-PAGE (right panel) and immunoblotted with anti-V5 antibody, pK. Arrows F1-F6 indicate heavy chain-dimers. (D) Cell lysates from (A), (B) and (C) were immunoblotted for MHC class I (pK), ERp57 and ERp72. Arrows indicate detected molecules as stated.

mutant (Figure 3.6A, arrow i) migrated with an apparent higher Mw than those detected for HLA- B*27:05wt and HLA-B27.C101S. This observation may suggest that an α 3-domain although not important for dimer formation, may contribute to the overall conformation of these structures.

C164 could simply participate in a C164-C164 disulphide linkage, or the removal of C164 could prevent other cysteine residues from participating in disulphide bond formation (Figure 3.7). In the absence of C164, C67 could form an intramolecular disulphide bond with C101, thus preventing intermolecular C67-C67 interactions (Figure 3.7). To address this possibility we mutated C67 together with C164 and analysed dimerisation as previously described. The HLA-B27.C67S-C164S mutant exhibited an enhanced ability to dimerise (Figure 3.6B and C, arrow F1). This observation suggests that mutating C164 does indeed perturb other cysteine-cysteine interactions and dimerisation can occur via C101. To determine whether C164 could participate directly in heavy chain-dimerisation, an HLA-B27.C67S-C101S mutant was generated and expressed in C58 cells. Immunoblotting revealed that HLA-B27.C67S-C101S was still capable of dimerisation (Figure 3.6C, arrow F4), confirming C164-C164 mediated dimerisation. Other minor high Mw populations can also be detected with the HLA-B27.C67S-C101S molecule forming a distinct series of higher Mw populations (Figure 3.6C, arrows F2, F5, F6).

The observed distinct high Mw species could represent HLA-B27 heavy chain in association with other molecules such as ERp57 or ERp72 that have previously been postulated to directly interact with MHC class I heavy chains (Lindquist et al., 2001) (Antoniou et al., 2002). To examine these possibilities, lysates were

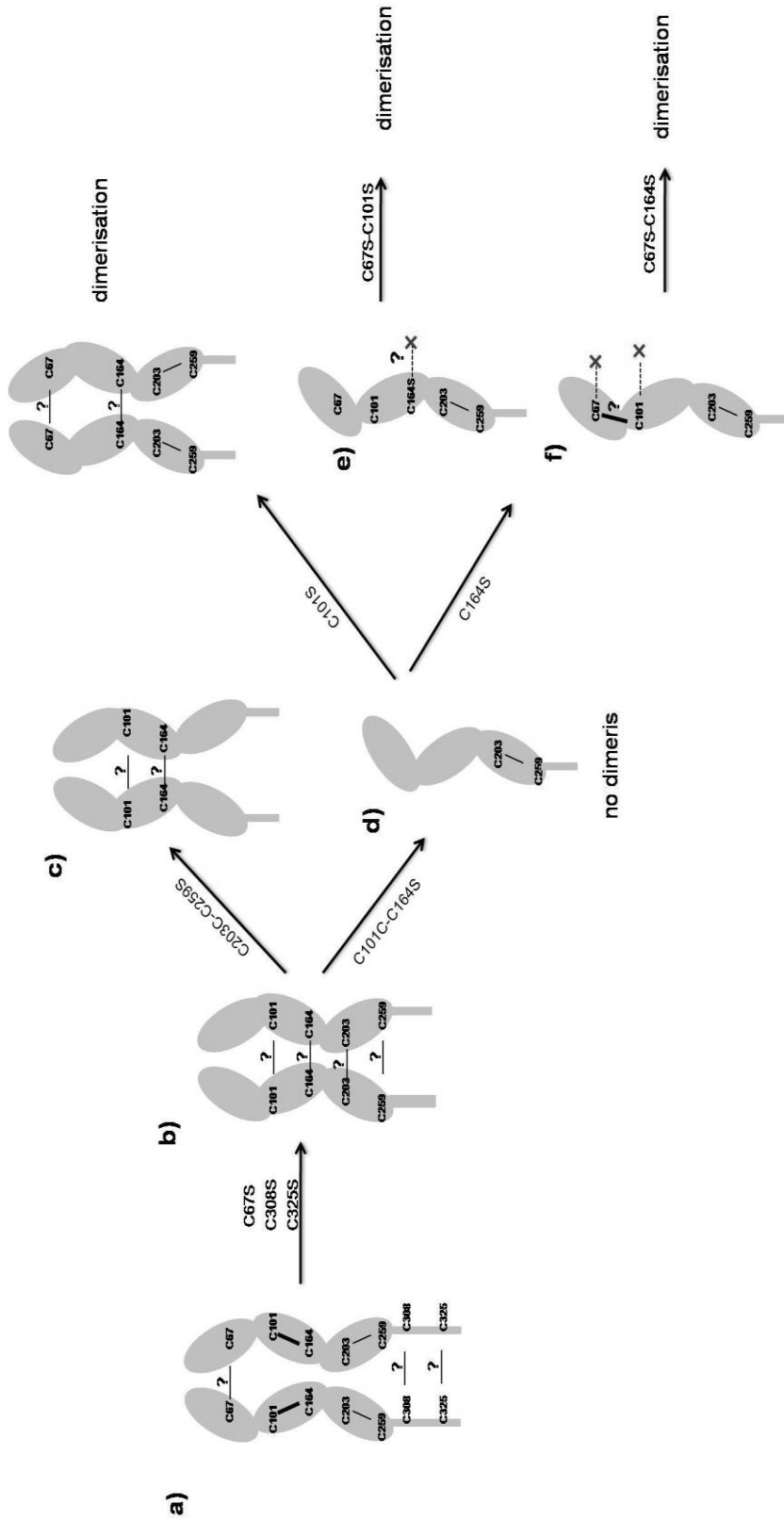


Figure 3.7. Analysing the contribution of cysteine residues in HLA-B27 dimer formation. a) Dimer formation could involve unpaired residues at p67, C308 and/or C325. b) In the absence of p67, C308 and/or C325 HLA-B27 can dimerise suggesting involvement of conserved cysteine residues. c) In the absence of C203-C259, heavy chain retains the ability to dimerise whereas d) mutation of C101-C164 diminishes dimer formation. C164S mutation could abrogate dimer formation due to e) abolished C164-C164 interaction or f) by preventing other cysteines from participating in interchain dimer formation. These possibilities were examined by introducing C67S-C101S and C67S-C164S respectively.

separated by non-reducing SDS-PAGE and probed for ERp57 and ERp72. However, none of the high Mw bands that were detected by immunoblotting with the anti-V5 antibody appeared to correlate with heavy chain species containing ERp57 or ERp72 (Figure 3.6D).

The differences observed between the α 2- and α 3-domain cysteine mutants in the dimerisation process could be the result of their different influence on heavy chain folding. To determine the influence of these structural cysteines on HLA-B27 folding I analysed the cell surface expression of these mutants by FACS analysis following 26°C incubation. It has previously been demonstrated that HLA cysteine mutants can be rescued by 26°C incubation. I postulated that if these cysteines had differential effects on folding, then cell surface expression could be differentially regulated following 26°C incubation. Both HLA-B27.C101S-C164S and HLA-B27.C203S-C259S mutants expressed in the C58 rat thymoma cell line were not detected at the cell surface by staining with the conformationally specific ME1 antibody at 37°C (Figure 3.8A). After overnight incubation at 26°C, cell surface expression of HLA-B27.C101S-C164S was not restored and only partially restored for C203S-C259S, suggesting that these molecules do not attain a fully folded state due to structural defects. However, the detection of ME1 reactive population at the cell surface following 26°C incubation of the C203S-C259S mutant does suggest that these molecules, even though generally unfolded/misfolded do have different folding properties. Impaired α 3-domain cysteine mutant folding could rise from inefficient association with β 2m which is require for efficient cell surface expression

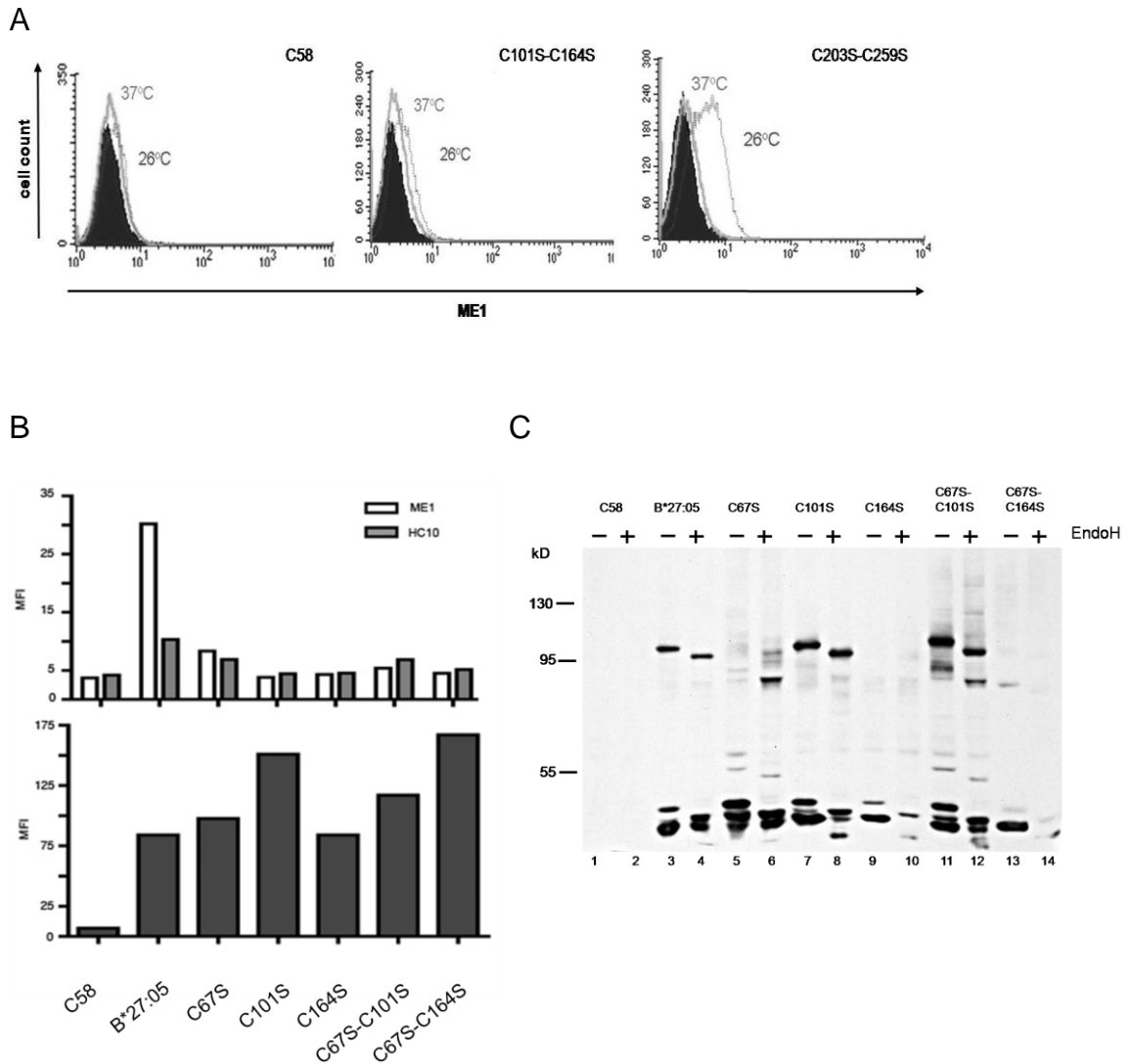


Figure 3.8. Structurally important C101 and C164 participate in B27 ER dimer formation. (A) Untransfected C58 and C58 cells expressing C101S-C164S and C203S-C259S were incubated overnight at 37°C or 26°C and stained for cell surface MHC class I expression with ME1. A representative from one of three experiments is shown. (B) C58 and C58 expressing B27, C67S, C101S, C164S, C67S-C101S and C67S-C164S were either stained for cell surface HLA-B27 with ME1 or HC10 (top panel) or for intracellular expression using the anti-epitope tag pK antibody (bottom panel). The mean fluorescence intensities (MFI) were plotted for each cell line. (C) Lysates from the cell lines from B were digested with endoglycosidase H enzyme for 1hr at 37°C, prior to analysis by non-reducing SDS-PAGE and immunoblotting with pK.

whereas the $\alpha 2$ -domain cysteine mutations could lead to misfolding within the peptide binding groove, thus preventing efficient peptide binding and further cell surface expression. Despite this significant folding failure the latter mutant does not dimerise.

I then examined the expression of the HLA-B27.C67S-C101S and HLA-B27.C67S-C164S mutants and detected very little if any of these molecules at the cell surface with the conformationally specific ME1 antibody, as well as the HC10 antibody (Figure 3.8B, top panel). The intracellular staining with the pK antibody revealed that protein levels of each cysteine mutant were expressed within two fold of each other. This observation suggests that apart from C67S, these mutants do not attain a fully folded state and thus reside within the ER (Figure 3.8B, bottom panel). To test if these heavy chain-dimer conformers were ER resident, lysates from each of the cysteine mutants were treated with Endo H that cleaves asparagine-linked oligosaccharide modifications attached to proteins soon after translation within the lumen of the ER. Endo H cleaves between two N-acetylglucosamine (GlcNAc) subunits directly proximal to the asparagine generating a truncated sugar molecule with one GlcNAc residue remaining on the asparagine. However, further processing of the protein through the Golgi apparatus modifies N-glycans making them resistant to Endo H digestion. Therefore, following digestion of the oligosaccharide modification with Endo H, a shift to a lower Mw when resolved by SDS-PAGE gel electrophoresis will be indicative that these protein species remain within the ER. (Figure 3.8C). 1 hour of Endo H digestion of the cell lysates from the previous experiment revealed that each of the high Mw conformers (Figure

3.8C, lanes 3, 5, 7, 9, 11, 13) were all sensitive to Endo H digestion (Figure 3.8C, lanes 4, 6, 8, 10, 12, 14) suggesting these dimer species are ER residents and do not attain a mature phenotype.

I have repeatedly observed that HLA-B27 heavy chain-dimers were comprised of different populations (Figure 3.5B and C, arrows i-iii). My data suggest that HLA-B27 dimerisation does not result in a single homogenous population, but can give rise to several dimer populations. It is possible that these HLA-B27 heavy chain dimer species could be rat cell line specific. Therefore to address this possibility, I compared dimer formation of HLA-B27 expressed in the human lymphoblastoid CEM cell line to that in the C58 rat thymoma cell line. To facilitate allele-specific detection of MHC class I heavy chains I used V5 epitope C-terminally tagged HLA-B*27:05 molecule. Initially, the C58.B27.V5 tagged expressing cell line was pretreated with NEM and lysates analysed non-reduced on SDS-PAGE followed by immunoblotting with both HC10 and anti-V5 epitope tag antibody pK. Immunoblotting revealed that both HC10 and pK antibodies could detect the same pattern of homodimer species (Figure 3.9A, left and middle panel, arrows i-iii). Similar examination of CEM.B27 V5 tag expressing cell line by immunoblotting with pK revealed that several dimer population could be detected in a human cell line (Figure 3.9A, right panel, arrows i-iii). Hence, the presence of distinct HLA-B27 dimer populations is not a rat cell line specific phenomenon.

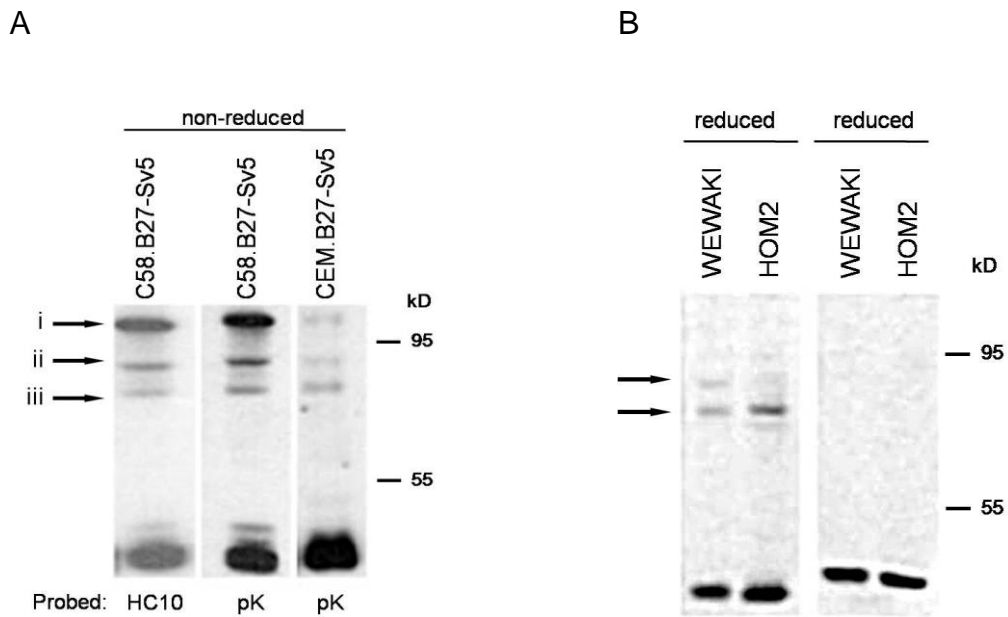


Figure 3.9. HLA-B27 heavy chain dimers are common to both rat and human cells. (A) C58.B*27:05 cells were alkylated with NEM prior to lysis and resolved by non-reducing SDS-PAGE, followed by immunoblotting with HC10 or pK and compared to lysates immunoblotted with pK from CEM cells transfected with B*27:05. Heavy chain-dimers are indicated by arrows i-iii. (B) Lysates of Human EBV cell lines WEWAKI and HOM2, expressing HLA-B27, were generated as in (A) followed by immunoblotting with HC10. High Mw structures are indicated by arrows.

To determine whether multiple heavy chain-dimeric bands could be detected at physiological levels, I immunoblotted lysates generated from two HLA-B27 expressing Epstein Barr Virus (EBV) transformed human B cell lines HOM2 and WEWAKI (Saleki et al., 2006). Immunoblotting with HC10 revealed multiple high Mw species (Figure 3.9B).

3.1.2.4 Composition of HLA-B27 heavy chain dimers

The variation in molecular size of different homodimers could be accounted for by different patterns of intermolecular disulphide bonds but this does not fully explain the presence of different HLA-B*27:05 homodimers species. Such differences could be explained by the following; (1) HLA-B27 forms distinct disulphide bonded conjugates with other ER resident proteins, (2) the heavy chain dimeric populations each exhibit a different redox state or (3) different redox states can be accompanied by changes in conformations.

It has been previously demonstrated that MHC class I heavy chains interact directly with the oxidoreductases ERp57 (Antoniou et al., 2002), ERp72 (Lindquist et al., 2001), PDI (Park et al., 2006) and the MHC class I specific accessory molecule tapasin (Chambers et al., 2008) (Santos et al., 2007). Moreover, these aforementioned interactions can engage both unpaired as well as conserved cysteines of MHC class I heavy chains and the aforementioned chaperones/oxidoreductases. In order to address the composition of these high Mw HLA-B27 conformers, non-reducing two-dimensional (2-D) gel electrophoresis was employed. Initially, proteins, following NEM pre-treatment, were separated according to their isoelectric point (pI) followed by separation

according to their molecular weight using non-reducing SDS-PAGE. If HLA-B27 dimers are composed of two identical B27 heavy chains they should have the same pI as the B27 monomer whilst the pI of B27 heavy chain conjugated to another protein would be altered. 2D-electrophoresis revealed several ~90kD bands (Figure 3.10, arrows i-iv). All of the detected dimer bands of B27 wt and C67S, C308S and C325S mutants have corresponding monomer bands (Figure 3.10, arrows M1-M3), indicating that these dimers are truly composed of two B27 heavy chains only. In accordance with previous observations, substituting C67 and C325 negatively affects the appearance of at least one heavy chain dimer population which is normally observed with B27 wt molecules (Figure 3.10, arrow ii compare to Figure 3.5B).

We can also observe that some monomer populations did not have or have very faint corresponding dimer bands (Figure 3.10, compare arrows M1-M3 with M4). A horizontal shift of these populations (Figure 3.10, arrow M4) on the gel indicates their more acidic pI value. It is likely that these bands represent HLA-B27 molecules that have exited the ER and undergone post-translational N-glycosylation modifications of the sialic acid attachment. Such a post-translational modification can occur mainly within the Golgi compartment, which has been reported to alter the pI of proteins towards a more negative net charge (Kumar et al., 2004). This observation would also suggest that the homodimers detected by 2D gel electrophoresis are mainly ER resident. The enhanced intensity of a negatively charged monomer band (arrow M4) in the case of B27.C67S suggests that the majority of the monomers acquire post-ER localisation. That is in accordance with previous findings, in which substitution

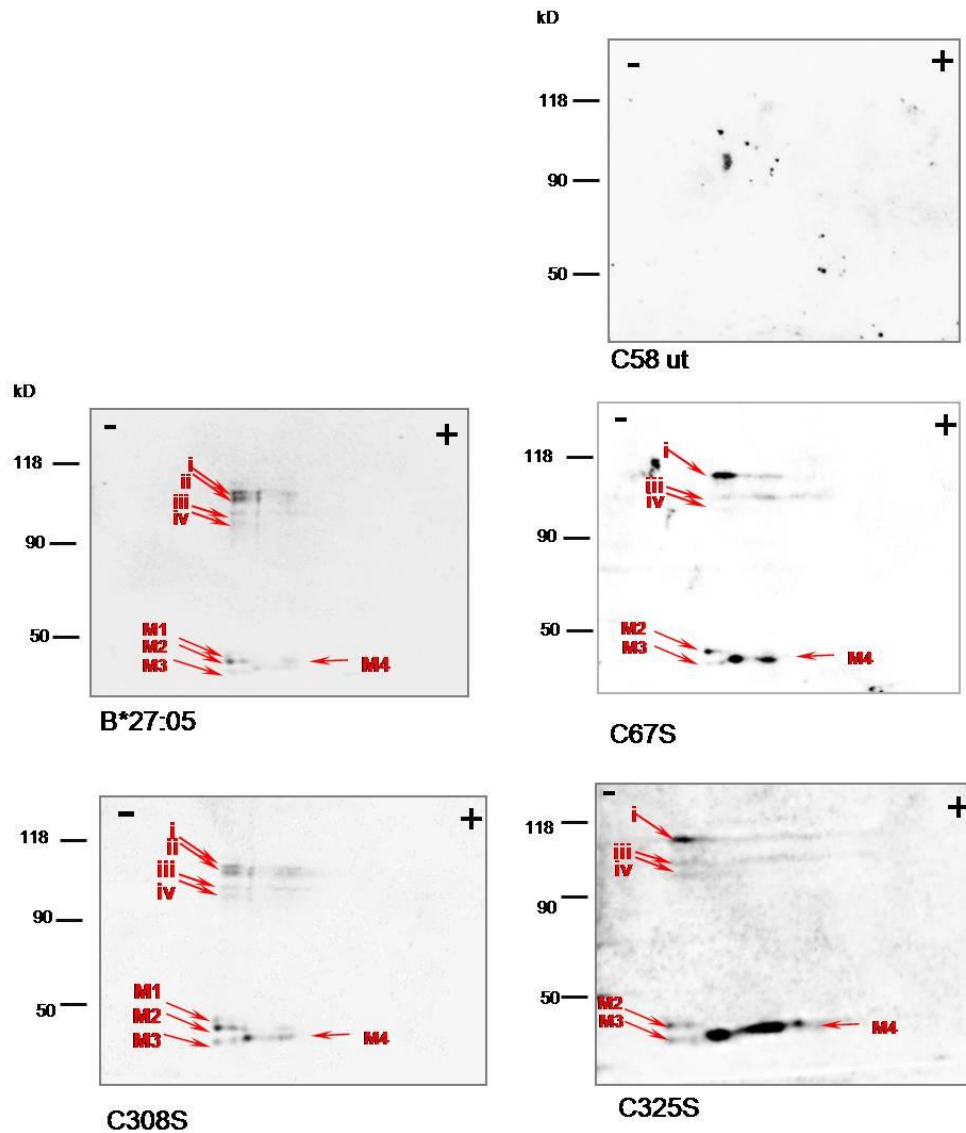


Figure 3.10. HLA-B27 dimers are composed of two class I heavy chains. C58 cells expressing B*27:05, C67S, C308S and C325S were pretreated with NEM, lysed and resolved non-reduced according to pI in the first dimension and non-reducing SDS-PAGE in the second dimension (2D electrophoresis). Each was analysed by immunoblotting with HC10. Arrows i-iv indicate detected class I homodimers bands whilst M1-M4 indicate the corresponding monomers.

of C67 to serine enhances egress of HLA-B27 from the ER, whilst the introduction of a cysteine at p67 in HLA-A2 slows down exit from the ER (Antoniou et al., 2004). Hence, the ability to dimerise appears to correlate with maturation rates of MHC class I molecules and the above data would provide further evidence for the hypothesis linking B27 slow folding kinetics and egress from the ER to an enhanced tendency to homodimerise.

3.1.2.5 HLA-B27 homodimers differ in the redox state

Knowing that HLA-B27 high molecular bands represent heavy chain homodimers I wanted to determine why these populations exhibited distinct molecular weight differences. The observed high Mw bands could be accounted for by distinct redox or folding states. The redox state of the protein refers to the net state of the sulphhydryl groups i.e. whether they are presented as thiols (reduced state) or disulphides (oxidised state), which in turn could influence protein conformations. The fully assembled MHC class I heavy chain conformation is characterised by oxidation of both structural $\alpha 2$ and $\alpha 3$ domain disulphide bonds whereas interfering in its oxidative folding may result in a degree of unfolding what can be monitored by a shift in its electrophoretic mobility (Dick et al., 2002) (Park et al., 2006) (Kienast et al., 2007). To monitor potential redox differences between these high Mw forms, I tested their susceptibility to reduction (Figure 3.11). C58.B27 cells were treated with increasing concentrations of dTT (0-20 mM) for 10 mins at 37°C before NEM alkylation and lysis in 1% NP40. Immunoblotting revealed that dTT treatment did not simply reduce all heavy chain dimers (Figure 3.12A). Each high Mw

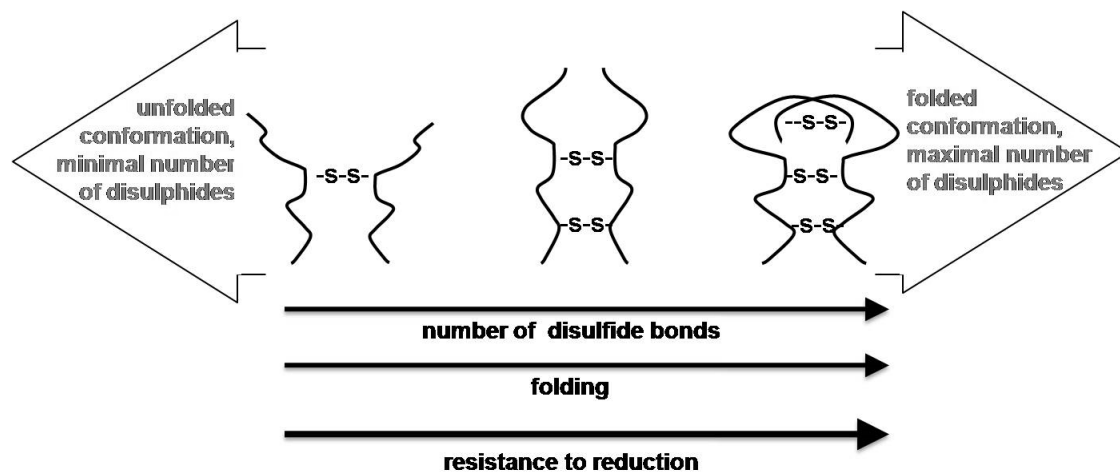


Figure 3.11. Protein susceptibility to reduction. The differential susceptibility of the molecules to reduction suggest that either the structure is held together by different numbers of disulphide bonds and/or have differentially exposed cysteine residues

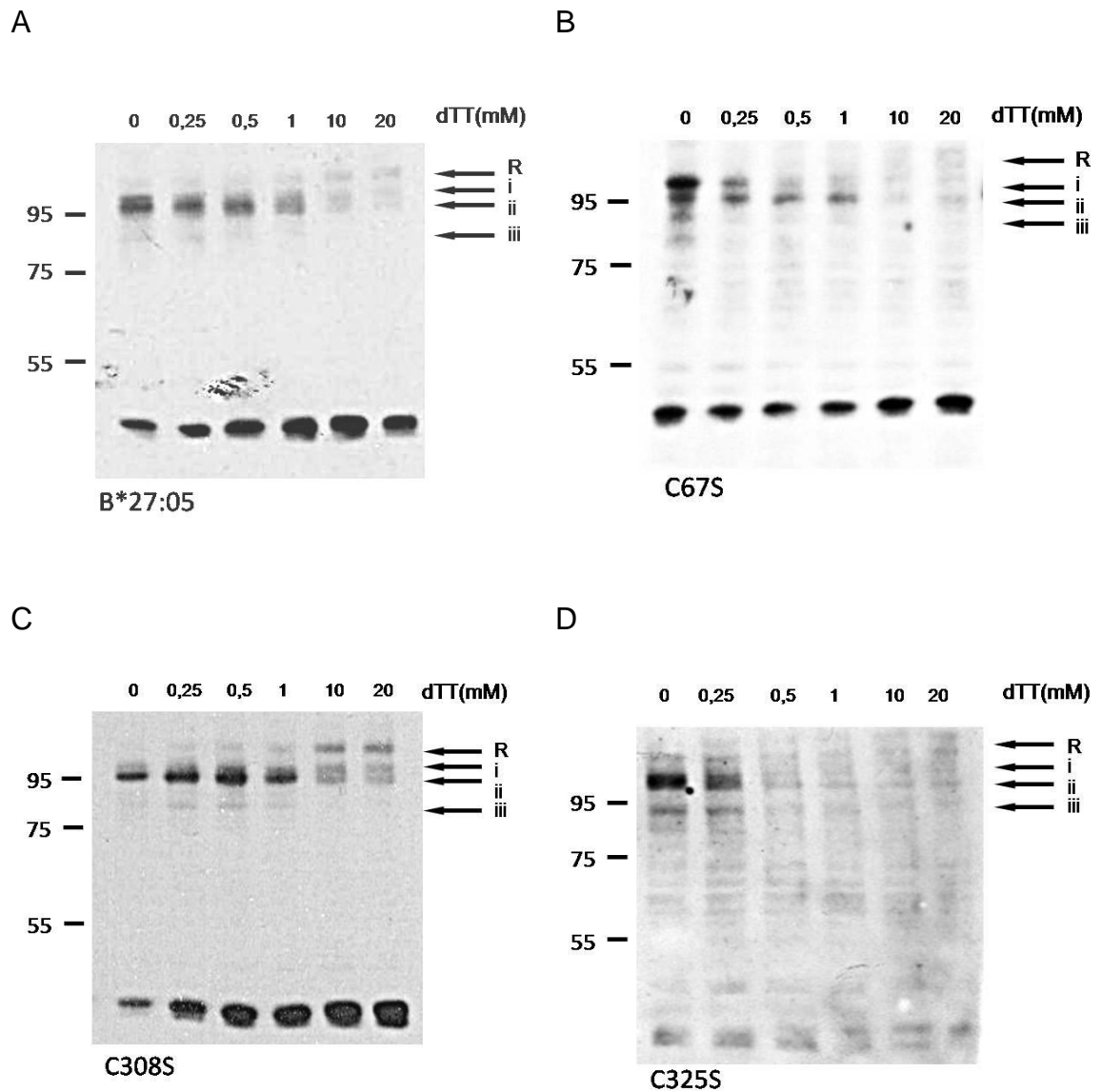


Figure 3.12. HLA-B27 heavy chain dimers exhibit differential susceptibility to dTT mediated reduction. Equal numbers of C58 cells expressing (A) B*27:05 wt and (B) B27.C67S, (C) B27.C308S and (D) B27.C325S were incubated with increasing concentrations of dTT (0- 20 mM) at 37°C for 10 mins, then NEM treated and lysed followed by non-reducing SDS-PAGE and immunoblotting with HC10. Heavy chain-dimers are indicated by arrows i-iii. Conformer of heavy chain-dimers, which appeared following reduction, is indicated by arrow R.

structure expressed by the HLA-B27wt molecule (Figure 3.12A, arrows i-iii) exhibits differential susceptibility to reduction. The homodimer band indicated by arrow iii was found to be the most susceptible to reduction (Figure 3.12A, arrow iii). The disappearance of bands iii and ii was accompanied by the appearance of an additional band (Figure 3.12A, arrow R). The prominent homodimer band of B*27:05 (Figure 3.12A, arrow ii), which is significantly reduced when C67 is mutated to serine (compare Figure 3.12A and B, arrow ii), is most resistant to reduction (Figure 3.12A, arrow ii). The homodimer population which seems to be the least affected by the absence of C67 (Figure 3.12B, arrow i) is more reduction-sensitive than the homodimer species depicted by arrow ii, as this population is susceptible to reduction following incubation with 0.5mM dTT (Figure 3.12B, arrow ii).

The differential susceptibility of the heavy chain-dimeric populations to dTT mediated reduction suggests these conformations are either held together by different numbers of disulphide bonds and/or have differentially exposed cysteine residues. The varying migration of the heavy chain-dimers in a denaturing gel could be due to differences in redox status or represent molecules with such redox states that have undergone conformational changes.

In this study, I analysed the role of the unpaired and conserved cysteines in the formation of HLA-B*27:05 ER resident dimers. I have shown that the unpaired C67 and C325 can participate in dimerisation but are not essential. Instead, my study strongly indicates a crucial role for C101 and C164 in HLA-B27 dimerisation. Furthermore, my data suggest that HLA-B27 dimerisation involves a preference or hierarchy of disulphide bonding. I propose that the predominant

disulphide bond within ER resident HLA-B27-dimers is mediated by C164-C164, followed by C101-C101, C67-C67 and with the possible involvement of C325-C325 interactions.

I also show that HLA-B*27:05 heavy chain-dimers are composed of multiple species, which are not conformationally equivalent and are possibly composed of folded and partially unfolded HLA-B27 molecules, which differ in their redox state.

3.1.3 Discussion

It has been postulated that the tendency for HLA-B27 to misfold and form homodimers may contribute to AS pathogenesis (Tran et al., 2004). Nevertheless, it remains elusive how these structures and aberrant forms occur and to what extent they participate in AS development.

In order to acquire the correct structure, molecules folding within the ER have to establish appropriate disulphide bonding, which requires cysteine residues to be both reactive and exposed. All MHC class I molecules possess 4 conserved cysteine residues which form two disulphide bonds within the $\alpha 2$ and $\alpha 3$ domain. Thus, not surprisingly we observed that mutation of the cysteine residues within the $\alpha 2$ and $\alpha 3$ domains in HLA-B27 disrupted the ability of the molecule to maintain an appropriate structure to an extent that it has become unrecognisable by conformational specific antibodies at the cell surface. Interestingly, HLA-B27 possesses a further 3 unpaired cysteine residues at position 67, 308 and 325, which are within the $\alpha 1$, transmembrane and cytoplasmic domains respectively. We observed that these residues can influence B27 folding, which subsequently has an effect on cell surface expression.

Much of the early studies of HLA-B27 dimerisation focused on the role of C67. Here I propose that the unique set of three unpaired cysteines at p67, 308, and 325, together within the HLA-B27 sequence could account for its unusual biochemical properties. I have confirmed findings from previous studies (Antoniou et al., 2004) (Bird et al., 2003) (Saleki et al., 2006) such that in the

absence of C67, HLA-B27 can still form high Mw disulphide bonded structures. Moreover, neither of the unpaired cysteines at p308 and 325 are critical for HLA-B27 homodimer formation, as B27 mutants containing substitution of these residues retain the ability to dimerise. Nevertheless I observed that C67 and C325 influence dimerisation to some extent, as at least one homodimer population was clearly influenced by these unpaired cysteines. It is possible that the B27 dimer species which are not apparent once C67 has been mutated to serine, represents cell surface dimers as detected by Bird et al (Bird et al., 2003).

To my knowledge this is the first study reporting involvement of C325 in B27 dimerisation although we cannot exclude that detected dimers are an exosomal-derived population. C325 is located in the cytoplasmic domain of HLA-B27, thus it is within an environment which has an overall reductive potential. Unlike the ER lumen, the cytoplasm tends to be unfavourable for disulphide bond formation. However, oxidative stress, which has been found to accompany inflammatory diseases (Sarban et al., 2005), can lead to thiol alterations that promote disulphide bonding within the cytosol (Thomas and Mallis, 2001) (Leichert and Jakob, 2004) (Cooper et al., 2002). Oxidative stress occurs when production of reactive oxygen species (ROS) exceeds the capacity of antioxidant defence mechanisms. ROS are highly reactive molecules containing oxygen and include nitric oxide, hydroxyl and superoxide radical anion. Reactive oxygen species, such as H_2O_2 that accumulate during oxidative stress, have the potential to oxidize protein sulfhydryls to protein disulphides (Thomas and Mallis, 2001) (Cooper et al., 2002). Interestingly, some studies have

reported that oxidative stress can be involved in AS pathogenesis (Stichtenoth et al., 1995) (Ozgoçmen et al., 2004) (Yazici et al., 2004) (Karakoc et al., 2007) (Stanek et al., 2010). That is manifested by increased concentrations of serum nitric oxide in patients with active spondyloarthropathy (Stichtenoth et al., 1995) or decreased activity of the antioxidant enzymes in plasma and erythrocytes in AS patients in comparison with healthy individuals (Stanek et al., 2010). Nevertheless, even in an unaltered environment, cytoplasmic cysteines have the potential to form disulphide bonds. Capps et. al has documented that mouse MHC class I alleles H-2L^d, D^d, and D^b can dimerise through an unpaired C340 within the cytoplasmic tail (Capps et al., 1993).

My studies suggest that C308 has the weakest influence on B27 dimerisation. This might be explained by the proximity of C308 to the ER membrane and the possible engagement of this residue in palmitoylation (Gruda et al., 2007). In this common post-translational modification, the palmitic acid is covalently attached to the thiol groups of cysteines preferentially located close to membrane domains (Bijlmakers and Marsh, 2003) and occupation of C308 by fatty acids could further prevent its involvement in aberrant disulphide bond formation between two B27 heavy chains. Alternatively, C308 could be engaged in disulphide interactions with tapasin, as suggested by in vitro studies by Chambers et al (Chambers et al., 2008). Since C308 is predominantly expressed in HLA-B alleles, but not in HLA-A alleles, this raises the question of how other non-HLA-B alleles would interact with the PLC, if they lack C308. Whether HLA-B27 does indeed interact with tapasin via C308 was not formally tested thus far.

I have demonstrated that B27 HC-dimers can be detected even in the absence of all three unpaired cysteines. These observations and those originally describing dimerisation with purified recombinant HLA-B27 strongly indicate that dimerisation will depend on the cellular and environmental context in which cysteines are exposed and not only to a specific set of thiols. My observations strongly suggest that conserved cysteine residues play a role in the dimerisation process. In our laboratory we originally identified a role for the structurally conserved C164 residue (Antoniou et al., 2004). In this present study I provide further evidence that C164 is indeed involved in the dimerisation process. It was originally proposed that the lack of dimerisation was due to the absence of C164-C164 disulphide interactions. However, in the absence of both C164 and C67, dimerisation was restored. One possible explanation would be, that by removing C164, the cysteines at p101 and p67 begin to preferentially participate in an intramolecular interaction with each other, thus preventing or reducing their ability to form intermolecular disulphide bonds. In the absence of both C67 and C164, the dimers that were detected presumably must represent heavy chain interactions via C101-C101. These observations therefore suggest both a novel role for C101 and C164 in HLA-B27 dimerisation (Figure 3.13).

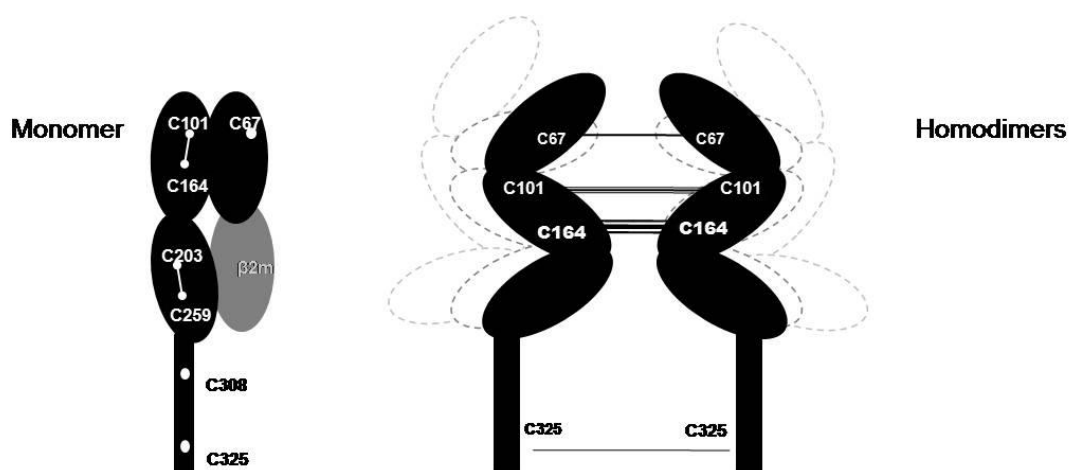


Figure 3.13. Scheme demonstrating the preference or hierarchy of disulphide bonds in HLA-B27 dimerisation. Fully oxidised MHC class I heavy chain monomer (left) contain two disulphides between C101-C164 and C203-C259. In HLA-B27 homodimer formation (right) C164-C164 is the dominant interaction, followed by C101-C101, C67-C67 and under certain conditions C325-C325 interactions can occur. Interchain disulphides engaging C101, C164 and C67 in varying orientations may also occur, but for simplicity these have been excluded. Homodimer conformers exist in different redox states that can be accompanied by changes in conformations (dotted lane).

Heavy chain-dimers observed with the C67S-C101S and C67S-C164S mutants could simply be due to the interaction between two misfolded molecules, that would otherwise not occur with intact HLA-B27 molecules. However, where both C101 and C164 have been removed and presumably HLA-B27 is unfolded, I was unable to detect dimerisation via C67-C67 interactions or any other C-C interactions. Molecular flexibility within the antigen binding groove of HLA-B27, which has been shown to allow the binding of the same peptide in two distinct orientations (Fabian et al., 2008) (Fabian et al., 2010) (Hulsmeyer et al., 2004) could contribute to such dimer formation described above. C101 and C164 could participate in heavy chain-dimerisation in the absence of dramatic unfolding events as these residues have been proposed to be partially oxidised until MHC class I molecules reach the PLC (Dick et al., 2002).

The lack of heavy chain-dimers in the absence of both C101 and C164 but not the C203 and C259 demonstrates that the $\alpha 3$ domain cysteines do not participate in dimerization. This observation is consistent with the idea that the C203-C259 bond is generated quickly and co-translationally, then rapidly buried and thus inaccessible within the immunoglobulin fold of the $\alpha 3$ -domain (Tector et al., 1997) (Ribaud and Margulies, 1992). The $\alpha 2$ -domain cysteine residues, form at a later stage in folding and could be maintained in a partially reduced/oxidised state until appropriate peptide binding takes place (Dick et al., 2002). In the case of HLA-B27, the proposed enhanced flexibility of the $\alpha 1$ - and $\alpha 2$ -domain could increase the likelihood for exposed cysteines to form dimers. If the C101-C164 bond forms at a later stage of the assembly process, partially folded/oxidised heavy chains could well be precursors to dimerisation. This

would explain the appearance of different heavy chain-dimer populations exhibiting different electrophoretic mobility profiles.

The differential susceptibility of heavy chain-dimer populations to reduction suggests that these conformers exist in different redox states. My study indicates that heavy chain-dimers are composed of multiple species, with each possibly exhibiting varying degrees of folding/unfolding. One possible explanation is the heavy chain-dimers which run with an apparent high Mw represents two molecules in an unfolded conformation perhaps including a degree of unfolding in the α 3-domain. This “open” conformation could dimerise via C101, C164 and C67 in varying orientations, resulting in up to nine disulphide bonds and thus accounting for an apparent enhanced resistance to dTT mediated reduction. Hence the species which are faster migrating and more sensitive to reduction, possibly represent heavy chains with a more folded conformation, probably with an oxidised α 3-domain and with fewer disulphide bonds. Whether the faster migration of some bands is indicative of a more oxidised and possibly compact molecular conformation could be examined by application of increasing oxidising conditions particularly using oxidising agents such as diamide that depletes the cell of reduced glutathione by targeting the thiols of GSH (Kosower and Kosower, 1995b). If B27 dimer species migrate according to their different oxidation states the population of very high Mw species should be more susceptible to increasing oxidative conditions. Furthermore, increasing the oxidation conditions should lead to the accumulation of the lower Mw populations representing compact oxidised species. Accordingly, the resistance of dimers species to increasing oxidation

conditions would suggest that these dimers are more 'compact' and/or oxidised in nature. Alternatively, these molecules maybe folded in such a manner that the cysteine residues are more exposed and susceptible to reduction.

I have shown that unpaired C67 and C325 can participate in dimerisation but are not crucial. I found that C164 is indeed important in ER HC-dimer formation but not only in mediating C164-C164 bond formation. Where heavy chain dimerisation was ablated by removing C164, I restored the ability of HLA-B27 to dimerise by removing both C164 and C67. Thus, my data suggest that C164 can be involved in a more complex series of redox reactions which allow for disulphide bonding between two HLA-B27 heavy chains. My data also suggests that ER resident dimers are not conformationally equivalent and are probably composed of folded and partially unfolded HLA-B27 molecules, which differ in their redox states.

3.2 Novel HLA-B27 conformation detected within the ER

3.2.1 Introduction and Aims

Redox regulation of proteins can play a key role in their ability to fold appropriately. Since exposure and reactivity of cysteine residues are a prerequisite for disulphide bond formation, I wanted to determine whether these characteristics correlated with HLA-B27 misfolding.

The redox state of both conserved (C101, C164, C203 and C325) and unpaired cysteine residues (C67, C308 and C325) within HLA-B*27:05 can influence the overall heavy chain conformation and folding properties. However, the number of cysteine residues cannot simply account for a particular redox state and/or its folding properties since most MHC class I molecules express different numbers of unpaired cysteines: from HLA-G and –A which possess one cysteine, HLA-B which possess 2-3 to HLA-C which can have up to 5 unpaired cysteine residues (Figure 3.14). Hence, there are several factors that potentially influence the degree to which particular cysteine residues influence the redox state of MHC class I heavy chains. The redox state of cysteine residues can be influenced by factors such as; i) the local environment of the cysteine residue i.e. whether it is expressed in the oxidising environment of the ER lumen that facilitates cotranslational disulphide bond formation, or the reducing environment of the cytosol, ii) the presence of surrounding residues which may influence cysteine thiol reactivity and iii) the system of oxidoreductases.

HLA	RESIDUES													
	1	42	67	99	101	164	203	259	308	309	321	325	339	340
B27	G	S	C	S	C	C	C	C	C	R	S	C	-	-
B14	G	S	C	Y	C	C	C	C	C	R	S	S	-	-
B39	G	S	C	Y	C	C	C	C	C	R	S	S	-	-
B7	G	S	Y	Y	C	C	C	C	C	R	S	C	-	-
B51	G	S	F	Y	C	C	C	C	C	R	S	S	-	-
A2	G	S	V	A	C	C	C	C	W	R	S	S	C	K
C	C	S	Y	C	C	C	C	C	M	C	C	A	A	C
G	G	C	T	I	C	C	C	C	W	R	-	-	-	-

α 1domain
 α 2 domain
 α 3 domain
transmembrane domain
cytoplasmic domain

Figure 3.14. Cysteine residues expressed by HLA-A, B, C and G allele. All MHC class I molecules express four conserved cysteine residues at positions (p) p101, p164, p203 and p259 forming two structural disulphide bonds (grey boxes). Different alleles express varying numbers of unpaired cysteine residues within their heavy chains.

These factors can all contribute to the overall folding properties of MHC class I molecules. The enhanced tendency for HLA-B*27:05 to form disulphide linked heavy chain dimers indicates that cysteines within B27 must be exposed and reactive.

Indeed, the sensitivity of cysteines to redox regulation can be influenced by proximity to polar and charged groups (Figure 3.15). Particularly, the basic amino acids such as lysine, histidine and arginine can accept protons from the sulphhydryl group leaving the cysteine side chain as a negatively charged thiolate ion ($R-S^-$) which is extremely susceptible to chemical modification:



Figure 3.15. Thiol reactivity can be influenced by the polar charged side chain of other residues. The transfer of a protonated form of the cysteine sulfhydryl group (-SH) to the side chain of a basic amino acid such as amine group (-NH₂) of lysine, results in the generation of an extremely reactive thiolate anion (-S⁻) and the protonated form of the basic amino acid.

As such, reactivity of C67 within the HLA-B27 sequence was proposed by Whelan et al. to be influenced by the adjacent lysine at p70 (Whelan and Archer, 1993). Regarding the reactivity of C67, adjacent residues at p66-68 could play a significant role as they are not only common for HLA-B27 but also two other alleles, HLA-B14 and -B39 which also exhibit an association with AS (Merino et al., 2008) (Merino et al., 2005). Although much of the attention has been dedicated to C67, the role of the unpaired cysteine residues C308 and C325 on the redox state of HLA-B27 has not been examined.

Moreover, since the main role of MHC class I molecules is the stable presentation of peptide to the immune system, the redox regulation of the peptide binding site within the ER seems to play a fundamental role in the biology of MHC class I molecules. The α2-domain disulphide bond (C101-C164) due to its location within the peptide binding groove, potentially can influence peptide binding and conversely, peptide acquisition could “protect” (cover) the disulphide bond and thus make it less susceptible to reduction. In line with the

first notion, mutation of C101 or C164 impairs optimal peptide loading and cell surface expression of HLA-A*02:01 (Warburton et al., 1994). On the other hand, blocking the delivery of peptides into the ER increases accessibility of the C101-C164 disulphide bond to reduction (Park et al., 2006). Hence, it seems that prior to optimal peptide binding, the α 2-domain disulphide is in dynamic equilibrium between an oxidised and reduced state. However, peptide binding may have the ultimate effect on both heavy chain conformation and cysteine accessibility, for instance by the requirement or use of tapasin (Williams et al., 2002). Whether this is accompanied by differences in the redox state was not determined. However, the employment of tapasin could have important implications for HLA-B27 biology, since two HLA-B27 subtypes, HLA-B*27:06 and B*27:09 that exhibit no association with AS, possess polymorphisms within a region described as responsible for a PLC-dependent/independent phenotype (Williams et al., 2002) (Park et al., 2003).

Hence, since the cysteine redox state influences the conformational changes within substrates, reliable and accurate methods to identify and quantify them are in high demand. *In vivo*, free thiols of cysteine residues are exceptionally prone to undergoing several reversible and irreversible modifications by redox active compounds such as GSH, ROS or reactive nitrogen species (RNS) (Barford, 2004) (Chouchani et al., 2011) (Cooper et al., 2002). This reactive nature of thiols makes them vulnerable to artifactual modification during protein isolation and labelling. However, the reactive nature of thiols can be exploited by strategies aimed at trapping the native redox state of cysteine thiols such as a “Rapid Acidification and Alkylation” assay used in this study (Figure 3.16).

To trap the native thiol/disulphide status the first and critical step in our experimental procedure is the rapid acidification with trichloroacetic acid (TCA) which protonates free thiols. Decreasing the pH of intracellular compartments can preserve both transient disulphide bond interactions and any exposed and potentially reactive thiol groups. Subsequent treatment of exposed thiols with the alkylating agent 4-acetamido-4'-maleimidylstibene-2,2'-disulfonic acid (AMS) can lead to the formation of cysteine-AMS conjugates, which due to the high Mw (~0.5kD) of AMS results in an increase in Mw of each non disulphide-linked cysteine residue, which can be monitored by non-reducing SDS-PAGE (Mezghrani et al., 2001) (Figure 3.16A).

Additionally, to determine how the reduced and oxidised molecules migrate on SDS-PAGE, prior to TCA treatment samples are pretreated with either the cell permeable reducing agent DTT or oxidising agent diamide. DTT is a strong reducing agent that breaks disulphide bonds within substrate protein(s) (Figure 3.17A). Protein susceptibility to DTT-mediated reduction is determined by the number of disulphide bonds and/or distinct cysteine residue exposure within substrate proteins. The action of diamide depletes the cell of reduced glutathione (GSH) by targeting the thiols of GSH (Figure 3.17B). Diamide induces disulphide bond formation between two GSH molecules which results in its conversion to an oxidized GSSG form (Kosower and Kosower, 1995b).

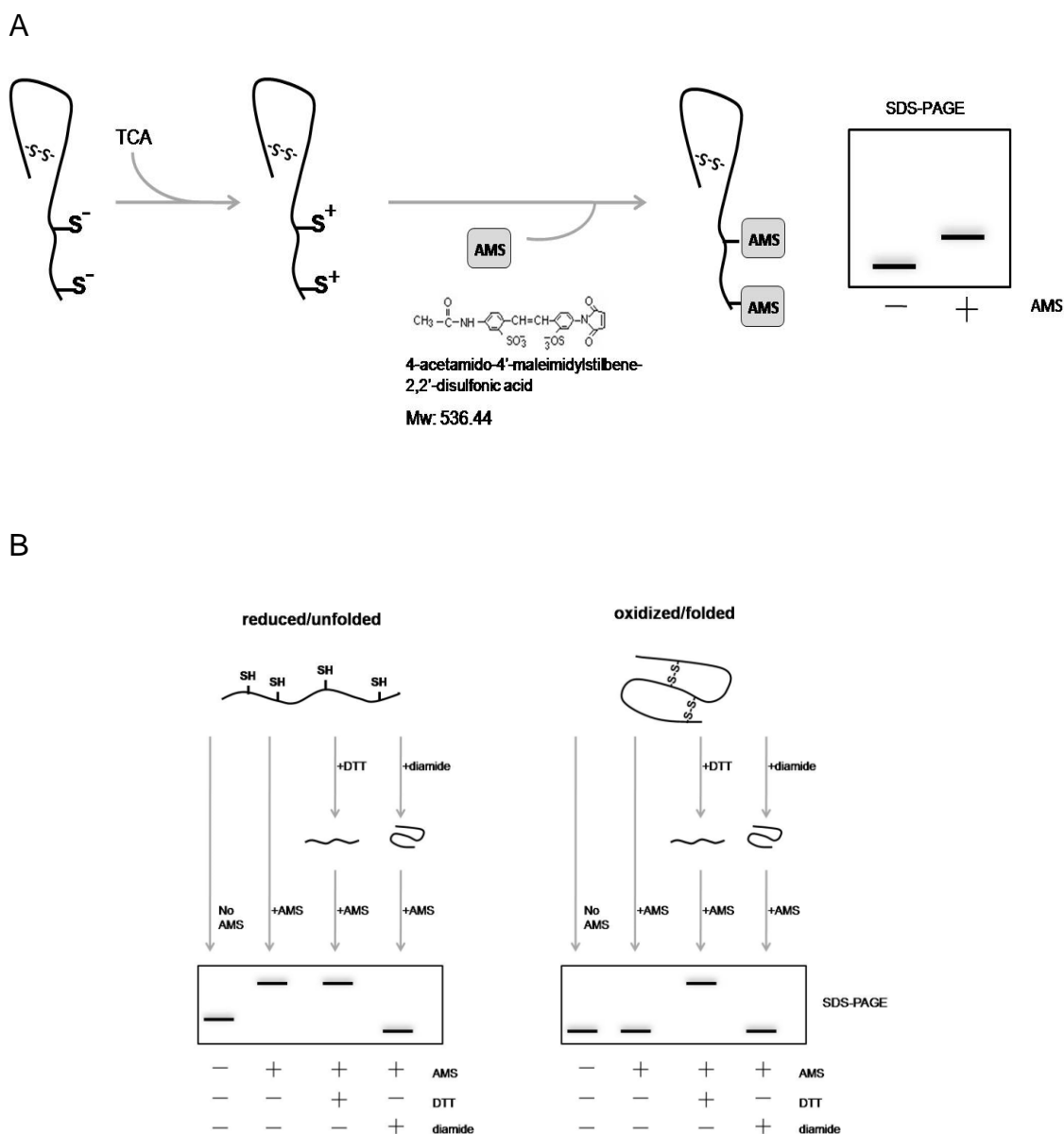
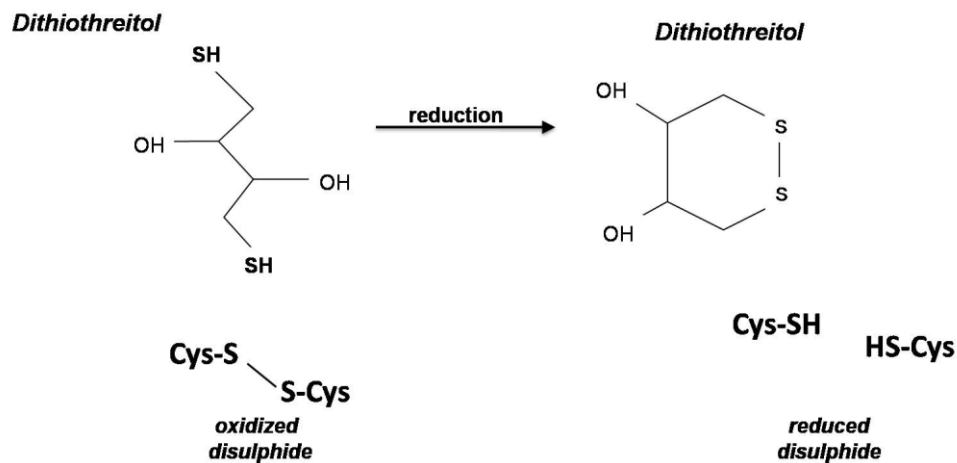


Figure 3.16 Scheme of the rapid acidification and alkylation assay. (A) Free cysteine thiols not involved in disulphide bond interactions can be protonated with trichloroacetic acid (TCA). Alkylation by 4-acetamido-4'-maleimidylstibene-2,2'-disulfonic acid (AMS) leads to binding of AMS to protonated thiols which can lead to an apparent increase in the Mw of the protein due to the Mw of AMS (~0.5kD). That in turn can be monitored as a shift in Mw by non-reducing SDS-PAGE. (B) Application of the TCA/AMS assay to establish conformations of reduced/unfolded (left) and oxidized/folded (right) protein based on how a protein reacts to the reducing agent dithiothreitol (DTT) or the oxidizing agent diamide respectively.

A



B

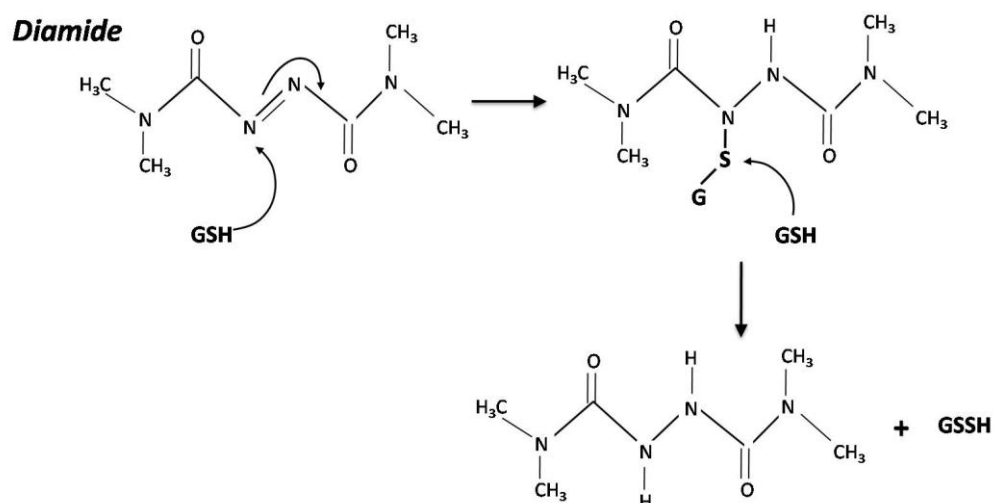


Figure 3.17. Redox reagents used in this study. (A) Dithiothreitol (DTT) is a strong reducing agent that breaks disulphide bonds within substrate protein(s). DTT contains two thiol groups. When one –SH group directly mediates reduction of protein disulphide, the second has a high propensity to close the ring, forming oxidized DTT and leaving behind a reduced disulphide bond. (B) Diazenedicarboxylic acid bis(N,N-dimethylamide) (diamide) is an oxidant that rapidly targets the cellular reduced form of glutathione (GSH). Diamide induces the formation of disulphide bonds in which intermediates of thiol-diamide are generated. Subsequent attack of the other GSH on the thiol-diamide adduct results in the formation of disulphide bonds between GSH forms, resulting in the generation of oxidised glutathione (GSSG).

Depletion of cellular GSH and an increase in the levels of GSSG further promotes disulphide bond formation within substrate proteins (Figure 3.17B).

The TCA/AMS assay has previously been successfully employed to monitor the redox state of oxidoreductases such as PDI, ERp57 and DsbA (Antoniou and Powis, 2003) (Fränd and Kaiser, 1998) (Mezghrani et al., 2001) and protein topology such as that of the sodium pump Na,K-ATPase (Lutsenko et al., 1997).

Aims

The aim of this chapter is to determine whether HLA-B27 heavy chain misfolding is due to the exposure of unpaired cysteine residues at p67, p308 and p325 and determine the redox state of the HLA-B27 heavy chain. Furthermore, I want to test whether the natural polymorphisms of non-AS associated HLA-B*27:06 and HLA-B*27:09 subtypes at p114 and p116 within the F pocket of the peptide binding groove, have an impact on HLA-B*27:05 cysteine accessibility.

3.2.2 Results

3.2.2.1 Rapid acidification and alkylation assay monitors MHC class I conformational changes

For cysteines to participate in disulphide bond formation and thus dimerisation, these residues must be exposed i.e. accessible and reactive. To determine whether the rapid acidification and alkylation assay ('TCA/AMS' assay) can be used to monitor the number and accessibility of free thiols within MHC class I molecules, I firstly employed this assay using two HLA-B*27:05 mutants in which either both cysteines within the α 2-domain (C101-C164) or α 3-domain (C203-C259) were substituted for serines. C58 rat thymoma cell lines expressing either C-terminal V5-tagged B27.C101S-C164S or B27.C203S-C259S mutants were initially incubated with either the reducing or oxidising agent DTT and diamide, respectively. Subsequently, to prevent artificial disulphide oxidation, the free thiols were protonated through cellular acidification with TCA followed by incubation in non-reducing sample buffer with or without the alkylating agent AMS. Samples were then resolved on non-reducing 8% SDS-PAGE and immunoblotted for MHC class I heavy chain with the anti-V5 antibody (Figure 3.18).

Following treatment of the B27.C101S-C164S mutant with AMS, the majority of the protein remained in an AMS resistant conformation (Figure 3.18, compare lane 1 and 2, arrow iii). This suggests that the C203-C259 disulphide bond is predominantly in an oxidized state. Incubation of the C203S-C259S mutant

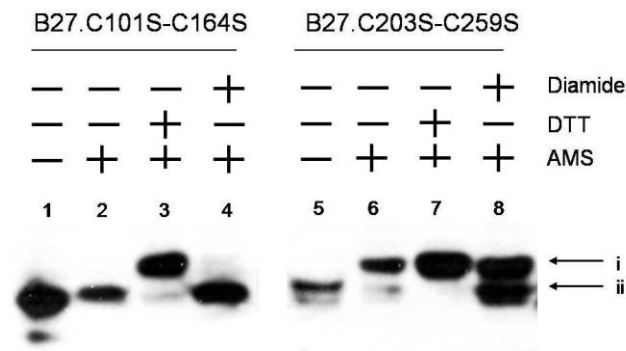


Figure 3.18. The rapid acidification and alkylation assay can be used to examine cysteine residue accessibility based on heavy chain conformations. C58 cells expressing B27.C101S–C164S and B27.C203S–C259S mutants were incubated overnight at 37°C (upper panel) or 26°C (lower panel) degree followed by DTT or diamide treatment and then were subjected to TCA/AMS analysis. Arrow i indicates reduced, AMS modifiable heavy chain form, ii-folded heavy chain.

with AMS resulted in most of the protein migrating as an AMS-modifiable conformation (Figure 3.18, compare lane 6 and 7, arrow ii) indicating that C101 and C164 within α 2-domain are accessible and reactive. Moreover, the B27.C203S-C259S mutant, following DTT-mediated reduction, resolved very similarly to the sample treated with AMS only (Figure 3.18, compare lane 7 and 6, top panel) suggesting that the lack of the α 3-domain cysteine residues results in the heavy chain adopting a predominantly reduced state. The above observations indicate that the α 2-domain and α 3-domain disulphide bonds are not equivalent. Furthermore the different conformational states of MHC class I molecules which result from these disulphide bonds can be monitored using the

TCA/AMS protocol. It appears that variations in accessibility of different cysteine residues within MHC class I molecules can depend on the protein conformation. Therefore the TCA/AMS assay as applied to MHC class I heavy chains can provide some information regarding the exposure of cysteine residues according to the conformation adopted by the molecule. However, the TCA/AMS assay as performed here cannot distinguish accurately the number of free cysteines.

3.2.2.2 HLA-B*27:05 adopts novel specific conformations within the ER lumen

I hypothesised that HLA-B*27:05 dimerisation and misfolding could be due to the exposure of cysteine residues within the heavy chain.

Knowing that cysteine accessibility and reactivity can be monitored by the TCA/AMS assay the cysteine exposure within HLA-B*27:05 stably expressed in the C58 rat thymoma cell line was determined. To determine how the redox state of HLA-B27 is influenced by unpaired cysteine residues at p67, p308 and p325 all three cysteines were substituted to serines (C67S-C308S-C325S). C58 cell lines expressing HLA-B27 wt and the B27.C67S-C308S-C325S mutant were pretreated with either DTT or diamide to determine the conformation of reduced and oxidised heavy chains respectively. Subsequently, cells were acidified with TCA followed by modification of free thiols with AMS (Figure 3.19A). Immunoblotting with HC10 revealed two distinct conformations/populations of HLA-B27 in the absence of AMS: the rapidly migrating heavy chain population depicted by arrow iii and a higher Mw species depicted by band iv (Figure 3.19A, lane 1, arrow iii and iv). On addition of AMS,

the low Mw form disappeared and coincided with the detection of higher Mw species represented by a faint band ii (Figure 3.19A, lane 3, arrow ii). On the contrary, the B27 population represented by band iii remained resistant to AMS modification suggesting that it represents a more compact heavy chain conformation in comparison with the low Mw species (Figure 3.19A, lane 1, arrow iv). The B27.C67S-C308S-C325S mutant also revealed more than one conformation at steady state (Figure 3.19A, lane 9), but these conformations resolved with different Mws when compared to those observed for HLA-B*27:05 wt. Notably the substitution of unpaired cysteines resulted in the lack of a low Mw species which was evident for HLA-B27wt (Figure 3.19A, compare lanes 1 and 9) indicating that the appearance of this band is dependent on C67 and/or C308 and/or C325. On addition of AMS to the B27.C67S-C308S-C325S mutant, the upper band in lane 9 appears to have adopted a slightly higher Mw (Figure 3.19, lane 11, arrow ii). Furthermore, the HLA-B27 wt molecule appears to be sensitive to DTT mediated reduction, indicating perhaps that the B27 heavy chain may be unstable (Figure 3.19A, lane 5, arrow i).

Mutation of the unpaired cysteine residues appears to result in a B27 heavy chain molecule which is even more susceptible to reduction (Figure 3.19, lane 13, arrow i) in comparison to the wt molecule (Figure 3.19A, compare lanes 3 and 13). This apparent enhanced sensitivity to reduction can be monitored not only by the level of the amount of reduced heavy chain following reduction but by also the amount of compact DTT resistant heavy chain.

To determine whether the detected conformations were cell surface or ER resident, TCA/AMS samples were digested with EndoH followed by immunoblotting with HC10 Ab. Sensitivity to Endo H digestion would be indicative of molecules not having acquired the appropriate sugar modification via transit through the secretory pathway to the cell surface. The conformations/populations that bound AMS in both B27wt, and B27.C67S-C308S-C325S mutant were mainly ER residents as they revealed sensitivity to Endo H (Figure 3.19A, compare lanes 3 and 4, 11 and 12). Similarly, the reduced heavy chain conformation of B27wt and the B27.C67S-C308S-C325S mutant seem to exist mainly in the ER compartment (Figure 3.19A, compare lanes 5 and 6, 13 and 14).

To determine whether the conformations detected by the AMS/TCA assay were unique to HLA-B27 or could be detected in other alleles, I tested another HLA-B transfectant (B7) and the endogenous alleles expressed in the following cell lines; HeLa, HEK293, CEM and NKR (Figure 3.19B). The TCA/AMS examination of HLA-B*0702 and endogenously expressed HLA products did not reveal conformations/populations similar to those detected for HLA-B27 (Figure 3.19B) suggesting thus far that these are B27 specific.

3.2.2.3 Influence of individual unpaired cysteine residues on HLA-B*27:05 redox state

The above experiment indicated that the unpaired cysteine residues can influence the redox state of the HLA-B*27:05 heavy chain. However, the contribution of the individual unpaired cysteine residues to the B27

conformation might vary at least due to their different localisation within the heavy chain or surrounding sequences as mentioned previously (Figure 3.14). Hence, C67, C308 and C325 were mutated individually and all the mutants were subjected to the TCA/AMS assay.

The lack of C67, C308 and C325 appears to lead to reduced detection of the rapidly migrating B27 conformation as depicted by band iv (Fig. 3.20 compare lanes 1, 5, 9 and 13). C67S and C325S exhibit reduced levels of an AMS-modifiable B27 conformation (Figure 3.20A, compare lane 2 with 6 and 14, arrow ii).

This analysis also revealed that mutating these unpaired cysteine residues can alter susceptibility of the heavy chain to DTT mediated reduction (Figure 3.20A, compare lane 3 with 7 and 15, arrow i). Substitution of C308 does not significantly change neither B27 heavy chain susceptibility to AMS-mediated modification (Figure 3.20A, lane 10, arrow i) nor sensitivity to reduction (Figure 3.20A, lane 10, arrow ii) in comparison with a B27 wt molecule.

Previously, we have used DTT reduction to demonstrate that HLA-B*27:05 is highly susceptible to reduction in comparison to other MHC class I molecules such as HLA-A*02:01 (Fussell et al., 2008). We postulated that this could be used to monitor the stability/compactness of the heavy chain at steady state levels within the ER. Also, as these mutants did exhibit differences in susceptibility to DTT mediated reduction, I therefore wanted to determine the influence of the individual unpaired cysteines to increasing concentration of DTT followed by AMS treatment (Figure 3.20B).

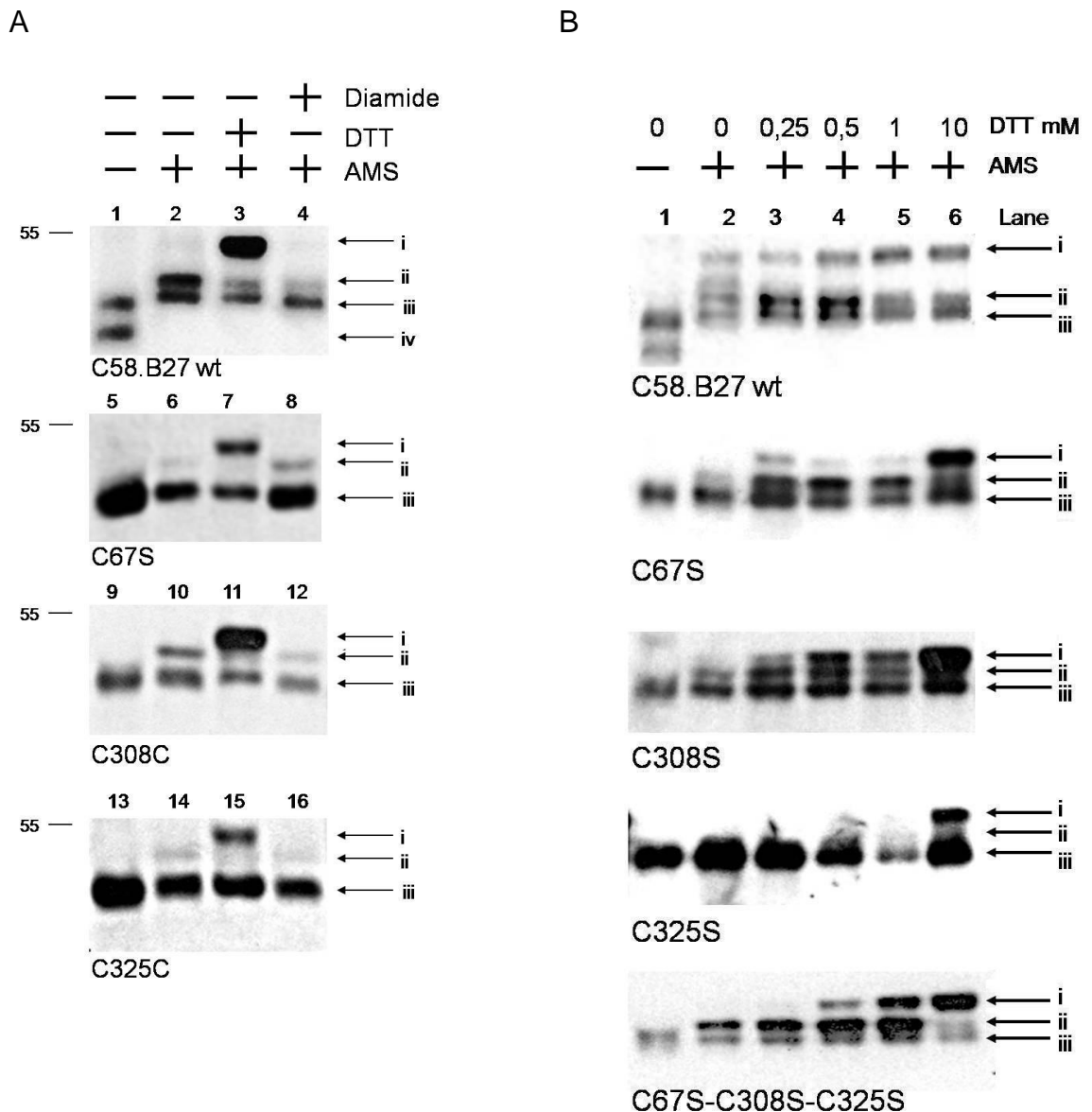


Figure 3.20. Unpaired cysteines residues differentially contribute to conformations adopted by HLA-B*27:05. C58 cells expressing HLA-B*27:05 wild type (wt) and C67S, C308S and C325S mutants were (A) either untreated or pretreated with oxidising (Diamide) or reducing dithiothreitol (DTT) agents or (B) with increasing concentrations of DTT, followed by TCA/AMS analysis. Band i corresponds to the reduced HLA-B27 heavy chain, band iv to a novel rapidly migrating conformation, iii to a compact folded heavy chain, whilst arrow ii indicates a novel conformation that was distinguishable upon addition of AMS.

Within all mutants, the increasing reducing conditions affected predominately the AMS-sensitive conformations (arrow ii), indicating that this conformation represents a less stable/unfolded heavy chain in comparison with the population depicted by band iii, which remains fairly resistant to reduction (Figure 3.20B). Interestingly, in comparison with B27wt, C67S and C308S, C325S displayed minimal AMS reactive material even at the highest dose of DTT (Figure 3.20B, lane 6, arrow i) suggesting that a lack of C325 seems to have a protecting effect regarding DTT-mediated reduction. Interestingly, removal of all three unpaired cysteines led to an increase in the amount of AMS-reactive conformations within the C67S-C308S-C325S mutant before reduction (Figure 3.20B, lane 2, arrow ii) indicating that the remaining conserved C101, C164, C203 and/or C259 must be accessible and reactive.

Taken together the above data indicate that HLA-B*27:05 exist in at least two conformations which differ in their redox state at steady state levels. The novel conformation depicted by arrow ii seems to exist in a partially folded state and its presence is influenced by unpaired cysteines at p67 and p325. Moreover, although the unpaired cysteine residues do not form structurally important disulphide bonds, they do appear to participate or contribute to the stability of the heavy chain.

3.2.2.4 Reactivity of C67 is influenced by surrounding residues

The TCA/AMS assays performed above revealed that C67 can (Figure 3.21A) have a profound effect on B27 to undergo AMS-modification (Figure 3.20A, arrow ii). To determine whether the presence of C67 which lies in the region of

the B pocket of the peptide binding groove (Figure 3.21A) can affect other HLA alleles in a similar way, I tested the non-AS associated HLA-A2 allele in which C67 was incorporated (Figure 3.21B). Rapid acidification and alkylation confirmed that HLA-A2 (Fussell et al., 2008), is resistant to AMS modification and DTT mediated reduction (Figure 3.21C lane 2 and 3), whereas the A2.V67C mutant revealed that introduction of C67 results in the HLA-A2 heavy chain being more susceptible to reduction (Figure 3.21C, lane 7, arrow i) and to a similar extent as that observed for HLA-B27 (Figure 3.20A, lane 3, arrow i). However C67 has only a modest influence on the appearance of the AMS-modifiable band i.e. C67 does not lead to the appearance of a predominant AMS-modifiable band which is seen for B27 (Fig. 3.20A, lane 2, arrow ii). However some AMS-modifiable bands are evident (Figure 3.21C, lane 6, arrow ii).

The sensitivity of cysteines to redox regulation was previously proposed to be influenced by the proximity to polar and charged group. It has been previously reported that reactivity of C67, which is located within the B pocket of the peptide binding groove, was influenced by adjacent residues, particularly lysine at p70 (Whelan and Archer, 1993) (Figure 3.21A). Hence it raises the possibility that other residues in proximity to C67, particularly residues 66-68 which are common for B27 and other AS associate B alleles i.e. HLA-B14 and –B39, may influence C67 thiol reactivity (Merino et al., 2008) (Merino et al., 2005).

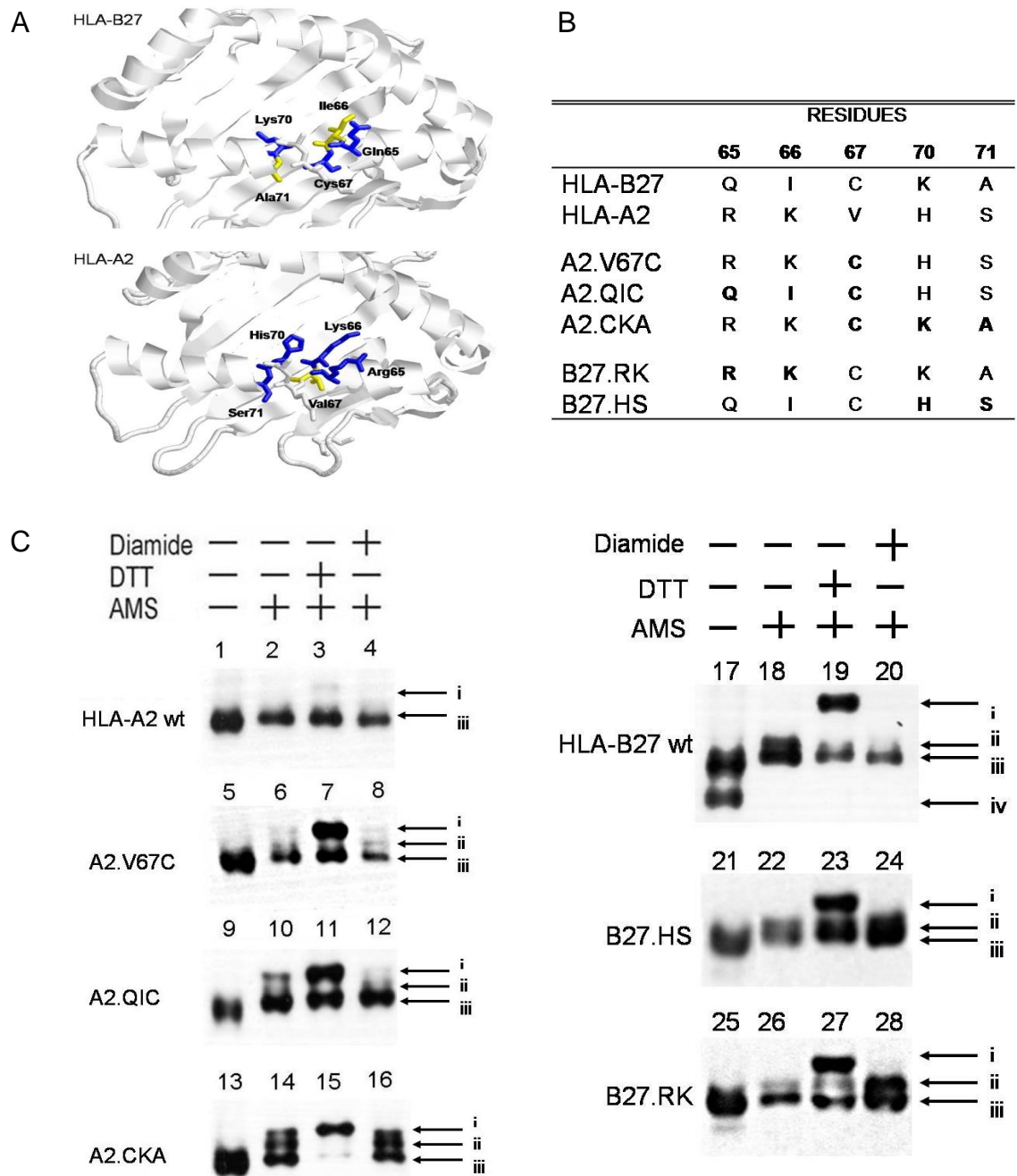


Figure 3.21. B pocket residues influence HLA-B*27:05 heavy chain redox state.

(A) Overhead view of the ribbon structure of the HLA-B*27:05 and HLA-A*02:01 peptide binding groove (PDB entry 2BST and 2X4N respectively). Side chains of the residues expressed at p65, p66, p67, p70 and p71 within the B pocket are displayed as sticks. (B) Comparison of the HLA-A2 and HLA-B27 sequence between residues 65-71 within both A2 and B27 B-pocket mutants. (C) TCA/AMS analysis of HLA-A2 wt in comparison with A2.V67C, A2.QIC, A2.CKA mutants (left) and HLA-B27 wt in comparison with B27.HS and B27.RK mutants (right). Arrows i indicate reduced heavy chain, ii-novel AMS modifiable form, iii-folded heavy chain.

To determine how surrounding residues can influence the reactivity of cysteine residues, A*02:01 cDNA was subjected to site-directed mutagenesis using the complementary primers that along with C67 introduced K70 and A71 into HLA-A2 sequence, creating mutant termed A2.CKA (Figure 3.21B). In the same manner A2 mutant mimicking B27 in the 65-67 region was generated (mutant A2.R65Q-K66I-V67C, termed A2.QIC) (Figure 3.21B). Each of these mutants were stably expressed in the C58 cell line and were subjected to the TCA/AMS assay (Figure 3.21C). The A2.QIC mutant revealed a very similar pattern to the A2.V67C mutant in terms of AMS-reactivity and susceptibility to DTT-mediated reduction (Figure 3.21C, compare lane 6 and 10, and 7 and 11 respectively). These observations indicate that the presence of Q65 and I66 do not significantly increase the reactivity of C67 residues (Figure 3.21C, compare lane 6 and 10). Notably, thiol reactivity within the A2.CKA mutant increased drastically, as demonstrated by a more apparent AMS-sensitive band (Figure 3.21C, lane 14, arrow ii) and also a dramatic increase in the sensitivity of the A2.CKA mutant to reduction (Figure 3.21C, lane 15, arrow i). This confirms that residues within the B pocket, particularly K70 can influence the reactivity of adjacent cysteine residues and potentially the folding properties of HLA-B27 (Dangoria et al., 2002) (Mear et al., 1999).

I then wanted to perform the converse experiments and examine the role of the B pocket sequences on B27 cysteine accessibility and reactivity. I therefore created B27 mutants mimicking the HLA-A2 sequence at p66-p65 and p70-p71 termed B27.RK and B27.HS respectively (Figure 3.21B). Hence, notably B27.HS and A2.QIC as well as B27.RK and A2.CKA share the same sequence between p65 to p71 (Figure 3.21B).

This reverse substitution introducing B pocket residues of HLA-A2 into B27 demonstrated that both B27.HS and B27.RK mutants remain accessible to AMS modification (Figure 3.21C, arrow ii, lane 22 and 26) and susceptible to DTT mediated reduction (Figure 3.21D, arrow iii, lane 23 and 27) to the same extent as the HLA-B27 wt molecule. The lack of a distinct influence of the p66-p65 and p70-p71 mutations on cysteine accessibility and reactivity within the B27 molecule could be due to the effect of other cysteine residues at p308 and/or p325. Taken together, the above data indicate that composition of residues within the B pocket of HLA-B27 contribute to heavy chain stability and cysteine accessibility/reactivity, however residues within this region are not the only factors contributing to conformations adopted by HLA-B27.

3.2.2.5 HLA-B*27:05 misfolding is influenced by peptide acquisition and residue at position 116

The above experiments indicate that the sequence within the HLA-B27 B pocket of the peptide binding groove can account for some of the enhanced susceptibility of the heavy chain to DTT mediated reduction. Indeed, MHC class I heavy chain sensitivity to reduction was previously proposed to arise from suboptimal peptide loading and/or incomplete folding (Antoniou et al., 2002).

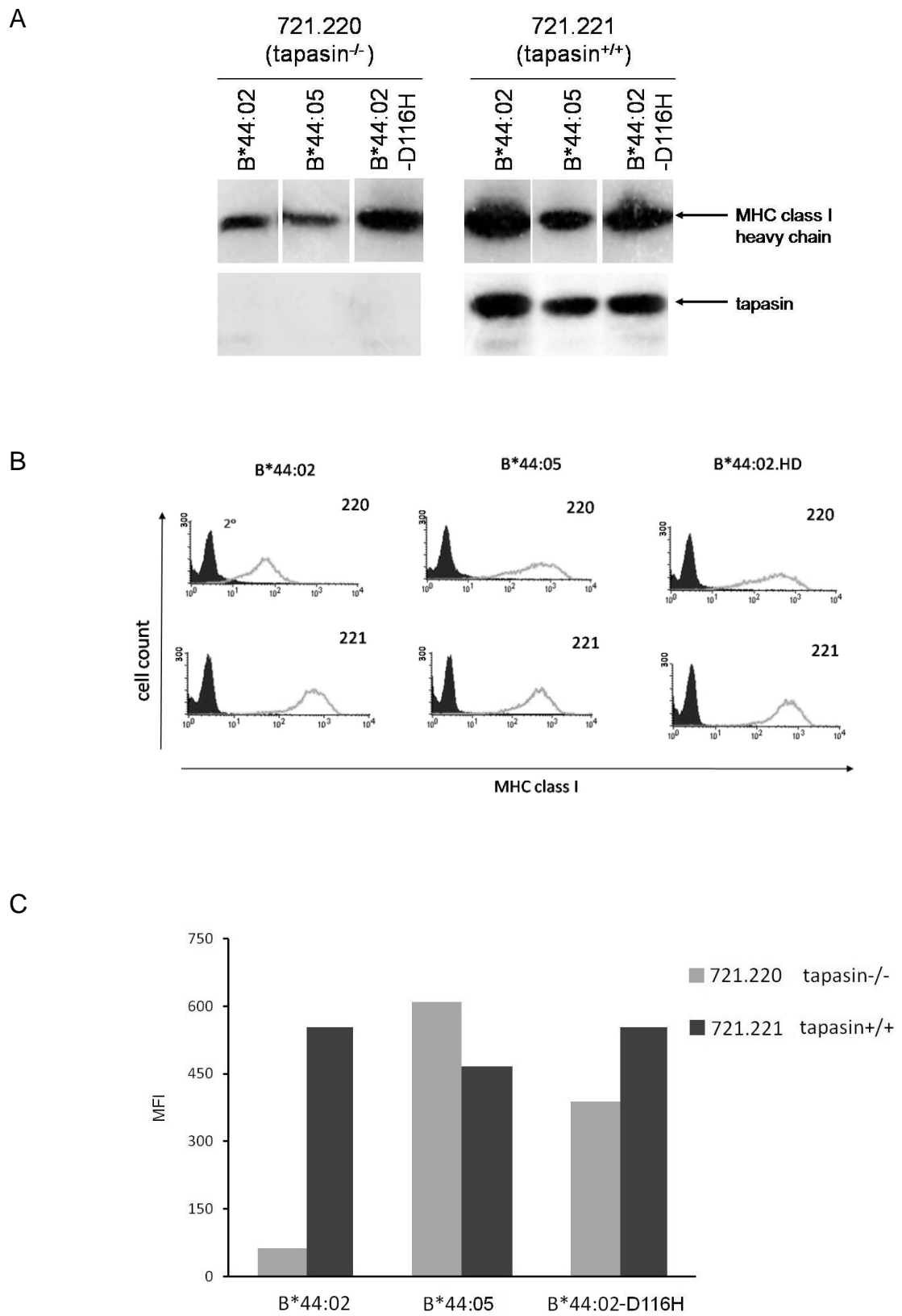
To determine whether conformational changes could arise through peptide binding I initially applied the rapid acidification/alkylation assay on the HLA-B*44:02 and B*44:05 subtypes which due to single amino acid polymorphism at p116 differ in their requirement for tapasin for the optimal peptide binding (see

“1.2.1.2.2MHC class I peptide optimisation”) (Peh et al., 1998) (Park et al., 2003) (Thammavongsa et al., 2006).

Hence, to determine the role of peptide content on the class I heavy chain, I analysed HLA-B*44:02 and B*44:05 expressed in both LCL721.220 tapasin-deficient and LCL721.221 tapasin-expressing cell lines, kindly provided by Prof Sebastian Springer, Jacobs University Bremen. Initially, to confirm that the HLA-B*44:02 subtype which expresses aspartic acid at p116 is highly dependant on tapasin for cell surface expression in comparison with HLA-B*44:05, which expresses tyrosine at p116, their cell surface expression was analysed by flow cytometry (Figure 3.22A).

Additionally, to test if differences between B*44:05 and B*44:02 alleles are solely due to the polymorphism within the F pocket I also analysed a B*44:02 mutant in which D116 was replaced with histidine, termed B4402-D116H. Within B*44:02, the presence of two adjacent aspartate residues at p114 and p116 are likely to be responsible for the specific ionic character within the F pocket since they might become strongly hydrated, which could cause conformational disruptions. Introduction of a histidine that is of similar shape to the tyrosine in B*44:05, but can undergo different interactions, into B*44:02, could imitate the environment within B*44:05 F pocket.

Firstly, the expression of HLA-B44 and tapasin was confirmed by immunoblotting of lysates from each cell line with HC10 (Figure 3.22A, upper panel) and anti-tapasin antibody (Figure 3.22A, lower panel), respectively.



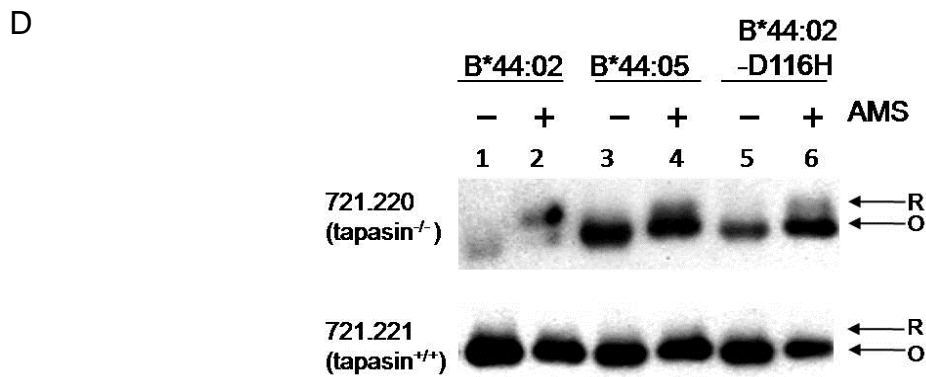


Figure 3.22. TCA/AMS assay can be used to monitor *in vivo* conformations depending on their ability to be loaded optimally using tapasin. (A) 721.220 and 721.221 cells expressing HLA-B*44:02, -B*44:05 and B*44:02-D116Y mutant were lysed in 1% NP40 detergent. Reduced lysates were resolved on an 8% SDS-PAGE followed by immunoblotting with HC10 (upper panel) and tapasin antibody (bottom panel). (B) Cells from A were analysed by flow cytometry for MHC class I cell surface expression using W6/32 ab. The filled curve is secondary antibody only control. (C) Bar chart representing Mean Fluorescence Intensity (MFI) of the data from B. (D) Cells from A were treated with DTT followed by TCA/AMS analysis and immunoblotting with HC10 antibody. Arrows R and O indicate reduced and oxidized HLA-B*44 forms respectively.

Cell surface staining with the MHC class I conformationally specific W6/32 antibody (Barnstable et al., 1978) confirmed that neither B*44:05 nor B4402-D116H required tapasin for efficient surface expression, whilst B*44:02 is poorly expressed at the cell surface in the absence of tapasin (Figure 3.22B and C). It also indicated that D116Y mutation reverses requirement for tapasin by HLA-B*44:02 (Williams et al., 2002) to a tapasin-independent phenotype (Figure 3.22B and C).

To determine how peptide can influence the conformation of the given HLA-B44 subtypes, the TCA/AMS assay was performed (Figure 3.22D). In the absence of tapasin, B*44:02 remained almost entirely in an “open”/accessible state as determined by AMS modification (Figure 3.22D, upper panel, arrow R, lane 2). However, this AMS modifiable band was not apparent when B*44:02 was expressed in the tapasin competent 221 cell line (Figure 3.22D, lane 2, lower panel), suggesting that tapasin can stabilise MHC class I heavy chain. In contrast, B*44:05 and the B*44:02-D116H mutant revealed a reduced tendency to bind AMS in both the absence (Figure 3.22D, lane 4 and 6, upper panel, arrow R) and presence of tapasin (Figure 3.22D, lane 4 and 6, lower panel, arrow R). Hence, the TCA/AMS assay can differentiate HLA conformations under conditions of optimal and suboptimal peptide loading.

To determine whether the direct presence of optimal peptide would influence cysteine exposure specifically within the HLA-B27 heavy chain, the TCA/AMS assay was then applied to a B27 Single Chain Trimeric molecule (see “3.3 The role of the PLC and peptide in HLA-B27 dimer *formation*”). In this instance the B27 heavy chain was physically linked to β 2m and a B27-specific influenza

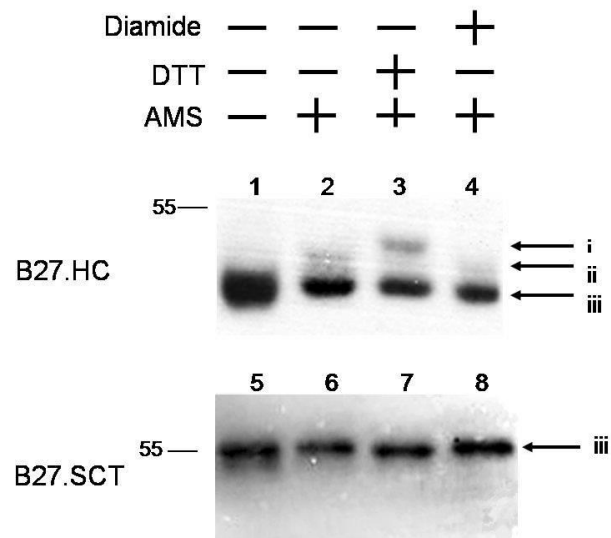


Figure 3.23. HLA-B*27:05 misfolding is influenced by peptide acquisition. HeLa cells stably expressing HLA-B*27:05 (B27.HC) and B*27:05 heavy chain linked to β 2m and peptide (B27.SCT) were either untreated or pretreated with the reducing agent dithiothreitol (DTT) or oxidising agent (Diamide), followed by TCA/AMS analysis. Arrow i corresponds to the reduced HLA-B27 heavy chain, arrow ii indicates a novel conformation that was distinguishable upon addition of AMS whilst arrow iii and iv to a compact folded heavy chain and SCT monomers respectively.

virus nucleoprotein [NP] peptide. TCA/AMS analysis of HeLa cells stably expressing two copies of either B27.SCT or B27 heavy chain only (B27.HC) (Figure 3.23) revealed that the presence of optimal NP peptide has a profound impact on B27 susceptibility to DTT mediated reduction and cysteine exposure. The B27.SCT molecule is less susceptible to reduction by DTT (Figure 3.23, arrow i) and AMS modification (Figure 3.23, arrow ii) respectively. Hence, sensitivity to DTT mediated reduction followed by AMS alkylation can be informative of conformational changes that follow acquisition of peptide.

The above experiments indicate that the residue at p116 influences MHC class I stability as determined by thiol accessibility. Following this observation I wanted to test how some of these residue differences at p116 could affect thiol accessibility of HLA-B27. Hence, B*27:05 was mutated to mimic the B*27:06 subtype as follow: H114D, D116Y, H114D-D116Y. Mutants, stably expressed in the C58 cell line, were subject to TCA/AMS analysis (Figure 3.24). The B27.H114D mutant revealed slightly enhanced susceptibility to reduction in comparison with HLA-B27wt molecule (Figure 3.24, arrow i). On the other hand, introduction of tyrosine at p116 decreased drastically the cysteine exposure of the B27.D116Y and B27.H114D-D116Y mutants, as demonstrated by the diminished accessibility to AMS modification (arrow ii). Strikingly, the fast migrating B27 conformation represented by arrow iv remained present regardless of any substitutions within the F pocket region. Taken together, the above data indicate that residue 116 within the peptide binding groove has a profound effect on HLA-B*27:05 cysteine accessibility.

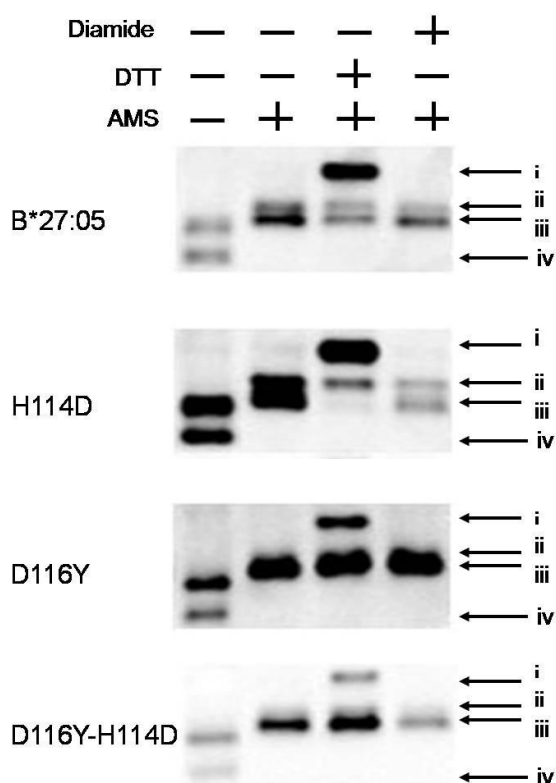


Figure 3.24. Residues 114 and 116 influence HLA-B*27:05 oxidative folding. C58 cell lines expressing HLA-B*27:05 wild type and a series of HLA-B27 mutants mimicking HLA-B*27:06 subtype, B27.H114D, B27.D116Y, B27.D116Y-H114D were pretreated with DTT reducing agent or the oxidizing agent diamide followed by TCA/AMS analysis. Arrow iv represents a fast migrating heavy chain conformation, iii- folded heavy chain conformation, ii- the AMS- modifiable heavy chain conformation, i- reduced heavy chain form.

3.2.3 Discussion

In order to acquire the correct structure, molecules have to establish appropriate disulphide bonding. As such, controlling the protein redox status is crucial for protein folding and assembly. Thus, the predisposition of HLA-B27 to form disulphide bonded heavy chain dimers could correlate with the tendency for this MHC class I heavy chain to misfold.

Using the rapid acidification/alkylation approach, I attempted to analysis certain aspects of the redox status of HLA-B27, namely the accessibility and reactivity of exposed cysteine residues. This approach has previously been used to determine the redox interactions between components of the MHC class I assembly pathway, namely ERp57 and tapasin or topology of transmembrane proteins (Antoniou and Powis, 2003) (Frand and Kaiser, 1998) (Lutsenko et al., 1997; Mezghrani et al., 2001) (Santos et al., 2007). I was able to detect two novel ER resident conformations of HLA-B*27:05 (Figure 3.20A, arrow ii and iii) which presumably had exposed cysteine residues. These conformations have yet to be identified for other HLA alleles. It is possible that these novel conformations could be precursors for HLA-B27 heavy chain-dimerisation.

The novel conformations/populations detected appear to depend on thiol accessibility. My data suggest that the unpaired cysteine residues within the HLA-B27 sequence exhibits differential reactivity or exposure. It seems that the presence of a reactive C67 and C325 within the HLA-B*27:05 sequence accounts for heavy chain instability measured by the sensitivity to reduction. TCA/AMS analysis indicates that introduction of C67 into HLA-A2 increases the

sensitivity of A2 to reduction what is further enhanced when C67 was expressed in combination with Lys70. The observed pattern of sensitivity to reduction of HLA-A2 B pocket mutants corresponded with their tendency to dimerise as previously demonstrated within our group (Antoniou et al., 2004). Hence cysteine exposure and heavy chain sensitivity to DTT mediated reduction, which I postulate is indicative of an enhanced tendency to misfold, correlates with formation of aberrant interchain dimers (Antoniou et al., 2004). On the other hand, introduction of the corresponding residues from the HLA-A2 B pocket into the HLA-B*27:05 did not significantly decrease heavy chain sensitivity to reduction. This confirmed that the presence of reactive C67 can not solely account for the HLA-B27 misfolding phenotype.

The thiol reactivity of C67 was previously examined (Whelan and Archer, 1993), however, this study was the first to report reactivity of the unpaired C325. It is an unexpected observation since C325 is not only located close to the C-terminal end of the heavy chain, which therefore places it within the reducing environment of the cytosol, but also is not surrounded by any positively charged amino acids that could alter its thiol reactivity. However, I can not exclude that charge interactions with other neighbouring amino acids could result in the generation of a reactive thiolate anion at C325. Although it is unusual for the cytoplasmically located residue to have an effect on the entire molecule, Capps et al. reported that the mouse MHC class I H-2L^d allele can dimerise through an unpaired cysteine within its cytoplasmic domain (Capps et al., 1993). Moreover, an H-2L^d mutant that also has a cytoplasmic domain unpaired cysteine but expressed at a different position lost the ability to form heavy chain dimers

(Capps et al., 1993). Therefore this observation would suggest that despite localisation within the reducing environment of the cytosol, cysteine residues can be exceptionally reactive and that cysteine reactivity could be determined by surrounding residues.

In comparison with C67 and C325, the cysteine at p308 located at the edge of the transmembrane and cytoplasmic domains, when not expressed, increases heavy chain sensitivity to reduction indicating its role in maintaining heavy chain stability. C308 is quite common among other HLA-B alleles (Figure 3.14) suggesting that its role might be more conserved. Indeed, C308 is at the interface of the stop transfer sequence (R309, R310, K311) and has been proposed to be involved in other biological functions of MHC class I heavy chains such as interactions with tapasin (Chambers et al., 2008) or palmitoylation (Gruda et al., 2007). The latter, is the covalent attachment of fatty acid to the cysteine residues favourably localised close to the transmembrane domain. This post-translational modification increases transmembrane protein hydrophobicity and their membrane association (Bijlmakers and Marsh, 2003) which could explain the influence of C308 on HLA-B27 stability.

One way to interpret resistance to DTT mediated reduction is that it can reflect the compact nature of the fully folded heavy chain which is characterised by cysteine inaccessibility to chemical modification. The observed HLA-B*27:05 susceptibility to reduction could reflect misfolding or binding of suboptimal peptide (Antoniou et al., 2002). It has previously been postulated that the α 2-domain disulphide bond remains partially reduced until loaded with optimal

peptide (Antoniou et al., 2002). In support of this hypothesis, in the absence of tapasin-mediated optimal peptide loading, HLA-B*44:02 heavy chain remained vulnerable to reduction unless tapasin was present. On the contrary, the tapasin-independent HLA-B*44:05 subtype remained stable in the presence and absence of tapasin and the stability was associated with polymorphisms at p116. Hence, the presence of tapasin promotes the adoption of a stable B*44:02 conformation but not B*44:05 and is regulated by polymorphism at p116.

However, my analysis does not demonstrate categorically that it is the C101-C164 bond that mediates these changes. Molecular dynamic studies performed by Garstka et al. suggest that requirements for tapasin-assisted peptide binding in the case of HLA-B*44:02 results from the fact that in comparison with HLA-B*44:05, the peptide binding cleft of empty HLA-B*44:02 is flexible and disordered and requires stabilisation by tapasin for efficient optimal peptide loading (Garstka et al., 2011). Since, substitution of D116Y within HLA-B27 makes the heavy chain more resistant to reduction, analysis of HLA-B44 subtypes allows me to hypothesise that it might be due to a similar molecular mechanism i.e. that substitution at p116 alters the flexibility of the HLA-B27 peptide binding groove. This in turn could potentially lead to faster peptide acquisition and a more stable α 2-domain conformation. This is in accordance with our own previous observations that B27.D116Y exhibits much faster folding kinetics than HLA-B*27:05 whereas the physical fusion of HLA-B27 with optimal B27-specific influenza-derived NP peptide leads to a more rigid, reduction resistant conformation. Hence these results suggest AMS-accessible

conformations which we observe for HLA-B27 may represent empty/suboptimally peptide loaded heavy chain in which the peptide binding region is disordered. Indeed, the molecular flexibility within the antigen binding cleft of HLA-B27, that allows for binding of the same peptide in two distinct orientations and accommodation of the extended peptides has been suggested previously (Fabian et al., 2008) (Fabian et al., 2010) (Hulsmeyer et al., 2004) (Madden et al., 1991) (Urban et al., 1994). This assumption could be formally confirmed particularly by the molecular dynamics analysis previously utilised to predict the flexibility of the HLA-B*44:02 peptide binding groove (Garstka et al., 2011). Here I provide evidence that this non-cysteine residue of the F pocket has a dramatic effect on cysteine exposure and accessibility since D116Y substitution results in “closed”/“compact” HLA-B27 heavy chain conformation.

In summary, HLA-B27 can adopt novel *in vivo* conformations at steady state which are characterised by prolonged cysteine residue exposure within the ER. In addition these exposed cysteines may contribute to the enhanced susceptibility to misfold, which was proposed to drive development of AS by activation of the UPR (Turner et al., 2005). The residues within the B and F pocket region of the peptide binding groove, particularly the reactive C67 and D116 can influence cysteine residue accessibility. Furthermore, enhanced HLA-B27 susceptibility to misfolding may be influenced by peptide acquisition which might be impaired due to an unstable peptide binding groove.

3.3 The role of the PLC and peptide in HLA-B27 dimer formation

3.3.1 Introduction and Aims

In the previous chapter I have defined the role of cysteine residues in the HLA-B27 dimer formation process and I have demonstrated that the heterogenous population of the heavy chain dimers arise through redox differences. However it remains an open question as to how peptide influences the dimerisation process.

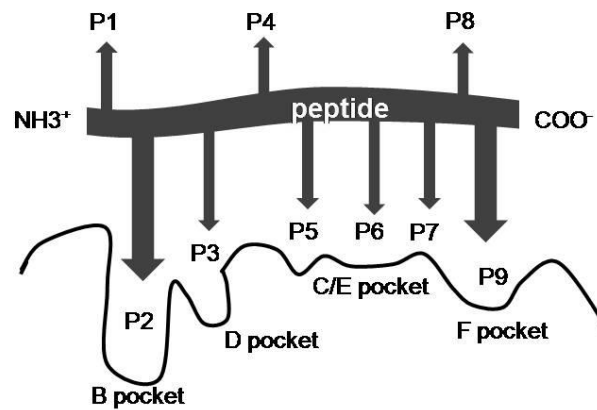
Natural polymorphisms between AS-associated HLA-B*27:05 and -04 subtypes and HLA-B*27:09, -06 and -07 that exhibit little or no correlation with disease are predominantly clustered within the F pocket region of the peptide binding groove. At the same time, the F pocket region influences the interaction with tapasin which plays a predominant role in peptide loading and optimisation of MHC class I heavy chains. Furthermore, numerous findings indicate that specific regions within the peptide binding groove might account for the unique folding profile of HLA-B27 (Mear et al., 1999) (Dangoria et al., 2002) (Antoniou et al., 2004). Hence, since peptide loading and the interaction with the PLC are major determinants of MHC class I folding, it gives rise to the possibility that peptide and/or peptide acquisition events occurring within the lumen of the ER may account or contribute to HLA-B*27:05 misfolding and its tendency to form heavy chain dimers.

Optimal peptide binding occurs within the PLC which is a multimeric complex which forms at the latter stages of MHC class I assembly (See Section 1.2.1.2.1 Composition of The Peptide Loading Complex) In the absence of TAP1 and TAP2, peptides generated by proteasomal degradation are not transported into the ER lumen. TAP deficiency does not prevent MHC class I heavy chain- β 2m associations but the lack of peptide delivery to the ER makes this complex unstable (Elliott et al., 1991). Interestingly, in the TAP-deficient (T2) cell line, neither folded or unfolded B27 dimers are present and restoration of TAP leads to the appearance of partially folded/unfolded (HC10-reactive) B27 heavy chain dimers (Dangoria et al., 2002), suggesting that peptide binding might play some role in HLA-B27 dimer formation possibly through the stabilization of the B27 heavy chain and prolonging the exposure of cysteine residues to the oxidising environment of the ER. Interestingly, association of heavy chain with the PLC have been directly linked with MHC class I assembly rates since a T134K single point mutation within HLA-A*02:01 abrogates its association with the PLC and correlates with faster trafficking through the ER in comparison with the wt molecule (Lewis et al., 1996). In turn, the slow folding rate of HLA-B27 has been correlated with its tendency to form heavy chain dimers (Antoniou et al., 2004).

Despite the much slower folding kinetics of the HLA-B*27:05 subtype in comparison with the non-AS associated -09 and 06 subtypes, once optimal peptide is acquired, HLA-B*27:05 seems to be more stable at the cell surface than non-AS associated subtypes (Galocha and Lopez de Castro, 2010). These observations suggest that peptide loading might be more rigorous in the case of HLA-B*27:05 thus accounting for the apparent slow folding phenotype.

The optimal MHC class I bound peptide has the correct length that makes maximal numbers of conserved hydrogen bonds and has the appropriate canonical anchor residues (Figure 3.25A). However, the optimisation process is an intrinsic property of the heavy chain, particularly the structure of the peptide binding groove. The exchange of suboptimal, low affinity peptide for optimal peptide is facilitated by the cornerstone component of the PLC specific accessory molecule tapasin (Dick et al., 2002) (Antoniou and Powis, 2003). However, some alleles can edit their peptide cargo regardless of their association with the PLC (Williams et al., 2002) and this seems to strongly correlate with a faster assembly rate (Neisig et al., 1996) (Blanco-Gelaz et al., 2009) (Goodall et al., 2006). The tapasin-dependency phenotype of MHC class I alleles strongly correlates with residues at p114 and p116 of the heavy chain which form part of the F pocket of the peptide binding groove (Williams et al., 2002). The F pocket accommodates the C-terminal anchor motif of the MHC class I associated peptides and can, along with the B pocket, define the peptide repertoire that binds to MHC class I heavy chains (Figure 3.25A and B). Interestingly, natural polymorphisms within the F pocket exist between AS and non-AS associated subtypes of HLA-B27 (Ramos et al., 2002b) (Varnavidou-Nicolaidou et al., 2004) (Olivieri et al., 2007) (Cauli et al., 2007) (Gomez et al., 2006) (see Table 3). How these polymorphisms influence the association of particular subtypes with the PLC has not been clearly demonstrated (Goodall et al., 2006) (Blanco-Gelaz et al., 2009).

A



B

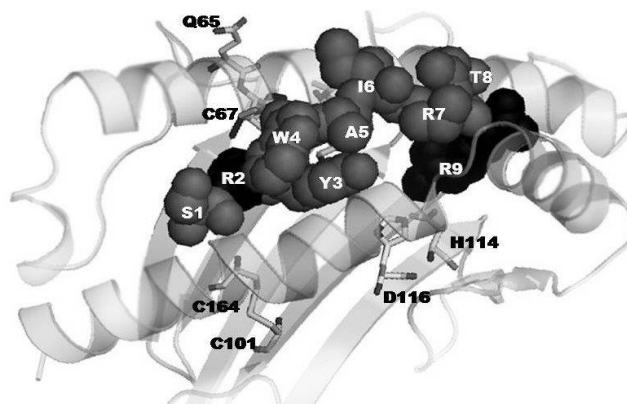


Figure 3.25. Peptide binding by MHC class I molecules. (A) Schematic representation of a nonamer peptide (P1-P9) binding the MHC class I peptide binding cleft which constitutes a B and F pocket accommodating side chains of the main peptide anchor residue P2 and P Ω (P9) respectively, and C, D and E pockets which accommodate secondary anchor residues. (B) Side view of the ribbon structure of HLA-B*27:05 peptide binding groove accommodating SRYWAIRTR influenza virus nucleoprotein peptide (PDB entry 2BST). C67 located within the B pocket, H114 and D116 making up the floor of the F pocket and C101-C164 disulphide bond are represented by sticks.

Table 3. Comparison of amino acids expressed within the F pocket region of differentially AS-associated HLA-B27 subtypes.

Allotypes	Residues		Tapasin dependence	AS association
	114	116		
B*27:05	H	D	+ / ++	+++
B*27:04	H	D	++++	++++
B*27:09	H	H	+++	+
B*27:06	D	Y	+	+
B*27:07	N	Y	+	++

Whether HLA-B*27:05 is tapasin independent is debatable. Initially HLA-B*27:05 was found to be relatively tapasin independent and able to self-optimize its peptide cargo over time since its cell surface expression was not significantly affected in the absence of tapasin (Williams et al., 2002). However, HLA-B*27:05 physically associates with the PLC (Peh et al., 1998) and in the presence of tapasin exhibits increased thermostability of heavy chain-peptide complexes, indicating that HLA-B*27:05 utilizes tapasin when present (Williams et al., 2002) (Kienast et al., 2007).

Goodall et al. demonstrated that HLA-B27 subtypes differ in their requirements for the presence of tapasin for cell surface expression, however there was no correlation between tapasin requirement and disease susceptibility (Goodall et al., 2006). Only HLA-B*27:04 was found to require tapasin in order to be efficiently expressed at the cell surface whereas other subtypes (05, 06 and 09) were also well expressed in a tapasin-deficient cell line. This, however, does not reflect the physical association of these subsets with the PLC since HLA-B*27:04, 05 and 09 but not 06 were incorporated into the PLC (Goodall et al., 2006). Interestingly, a lack or brief association of HLA-B*27:06 with the PLC correlated with the faster maturation rate and increased expression of B2m-free heavy chain at the cell surface as measured by staining with the HC10 antibody (Goodall et al., 2006). In general, alleles poorly associating with the PLC reveal faster folding kinetics and in the case of HLA-B*27:06 it seems to be at the price of efficient peptide loading. In this study HLA-B*27:05 and 09, which differ by only one residue at p116 (aspartic acid-to-histidine substitution), share the same characteristics regarding tapasin-dependency for cell surface expression and association with the PLC but yet are at the opposite ends of the spectrum

of AS-association. However, a different study by Blanco-Gelaz reported that HLA-B*27:09 demonstrated enhanced TAP association and even slower maturation rates in comparison with HLA-B*27:05 but also other subtypes such as 04 and 06. Thus we can speculate, that the 114-116 region could influence HLA-B27 structure and/or peptide binding, which could explain the differential subtype associations with the spondyloarthropathies. However, the influence of natural polymorphisms within the peptide binding groove of differently AS-associated subtypes on their assembly and folding is still to be determined.

Importantly, optimal peptide binding can induce conformational changes within the MHC class I heavy chain leading to a 10-fold enhancement in the interaction between heavy chain and β 2m (Elliott et al., 1991) whilst, peptide disassociation causes β 2m loss (Smith et al., 1992) (Smith et al., 1993). This is a potentially important consideration since the majority of aberrant heavy chain dimers within the ER lack β 2m as determined by immunoprecipitation with HC10-antibody (Dangoria et al., 2002). Furthermore, HLA-B27 was initially observed to form heavy chain dimers when refolded in vitro in the absence of β 2m (Allen et al., 1999). On the other hand, β 2m-deficiency alone leads to the development of spontaneous arthritis regardless of HLA-B27 expression in the mouse model and a lack of β 2m leads to the formation of heavy chain dimers of certain mouse MHC class I alleles (Capps et al., 1993) (Kingsbury et al., 2000). Hence, the absence of β 2m is a condition that in general induces heavy chain misfolding. Nevertheless, HLA-B27 dimerisation is unique since both β 2m-associated and β 2m-free B27 heavy chain dimers can form within cells with an intact assembly pathway (Dangoria et al., 2002).

Components of the MHC class I heavy chain- β 2m-peptide trimeric complex seem to co-operate and influence each other and account for stability. As affinity and availability of β 2m and peptide binding can influence the efficiency of MHC class I heavy chain folding, one of my experimental approaches was to therefore provide HLA-B27 heavy chain with the constant presence of β 2m and peptide by physically linking these three units. These normally separated components were joined by a flexible linker sequence in a single polypeptide chain referred to as a Single Chain Trimer (SCT) (Figure 3.27A). The SCT has been designed to improve the efficiency of MHC class I assembly for the purpose to enhance the CD8⁺ T cell response, in immunotherapeutic approaches such as vaccination against tumors and numerous infectious diseases (Huang et al., 2005) (Huang et al., 2007) (Hung et al., 2007) (Jaramillo et al., 2004) (Li et al., 2010) (Ordaz et al., 2010). There is clear evidence that SCT have an advantage over natural MHC class I- β 2m-peptide complexes in their stability at the cell surface (Primeau et al., 2005) (Yu et al., 2002) and in their ability to stimulate T cells as well as their potential to be recognised by NK cells (Brzostek et al., 2010) (Kim et al., 2005) (Crew et al., 2005). Due to the physical linkage of heavy chain- β 2m-peptide, it has been proposed that SCT would be chaperone independent, as demonstrated by their efficient expression in TAP deficient cell lines (Kotsiou et al., 2011). The physical linkage of peptide would obviate the requirement for peptide processing, optimization and exchange reactions. Testing HLA-B27 in the form of a Single Chain Trimer allowed me to determine the influence of peptide and B2m on HLA-B27 dimer

formation regardless of its propensity to misfold and interactions with ER chaperones.

Aims

The above data allowed me to hypothesise that HLA-B27 specific interactions of heavy chain with the PLC and/or altered peptide acquisition may account for its slow folding rate which could lead to dimer formation. Hence, this chapter aims to investigate the role of the antigen presenting pathway in HLA-B27 misfolding and dimerisation as well as the influence of β 2m and peptide in this process. I wanted to determine whether HLA-B27 polymorphisms within the peptide binding groove correlates with HLA-B*27:05 tendency to form aberrant heavy chain dimers.

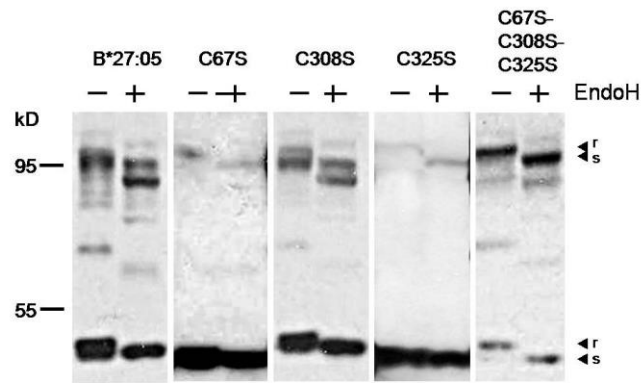
3.3.2 Results

3.3.2.1 HLA-B*27:05 dimerises within the PLC

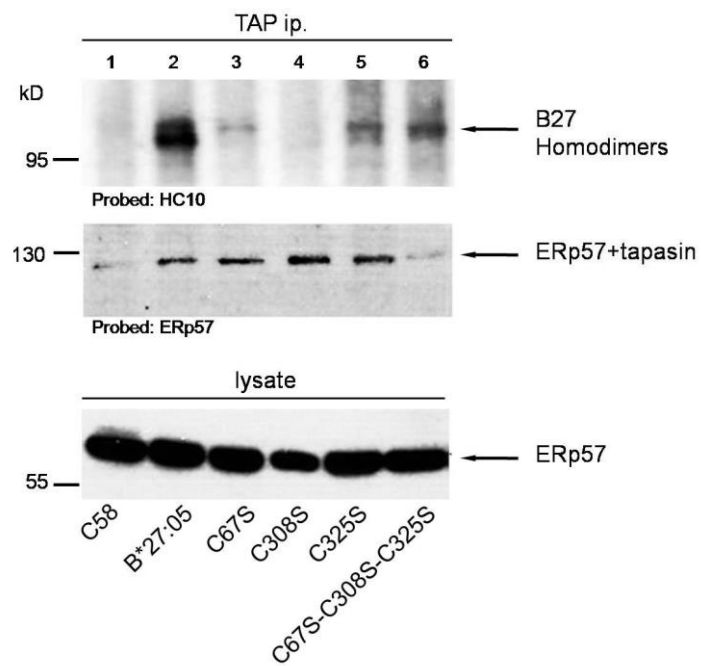
The majority of HLA-B27 dimers are located within the ER (Dangoria et al., 2002). Firstly to determine whether previously detected HLA-B*27:05 heavy chain-dimer conformers were ER or cell surface residents, I pretreated C58 cells expressing HLA-B27, B27.C67S, B27.C308S and B27.C325S with NEM and digested lysates with EndoH. Following SDS-PAGE electrophoresis and immunoblotting with HC10, there was indeed a change in M_w (Figure 3.26A, arrows) suggesting that most of the B27 dimeric populations were ER residents.

ER resident, HC10-reactive HLA-B27 dimers, were demonstrated to form shortly after heavy chain synthesis (Dangoria et al., 2002). The chaperones aiding the early MHC class I folding stage i.e. calnexin, BiP and the oxidoreductase ERp57, have been detected in enhanced association with HLA-B27, hence it was proposed that they might be involved in the dimerisation process (Antoniou et al., 2004). Diminished HLA-B27 dimerisation in the absence of TAP and the accumulation of dimers in the absence of tapasin, suggests a role for the PLC in B27 heavy chain dimer formation. To test the influence of the PLC on dimer formation firstly I analysed the interaction of HLA-B*27:05 with TAP.

A



B



C

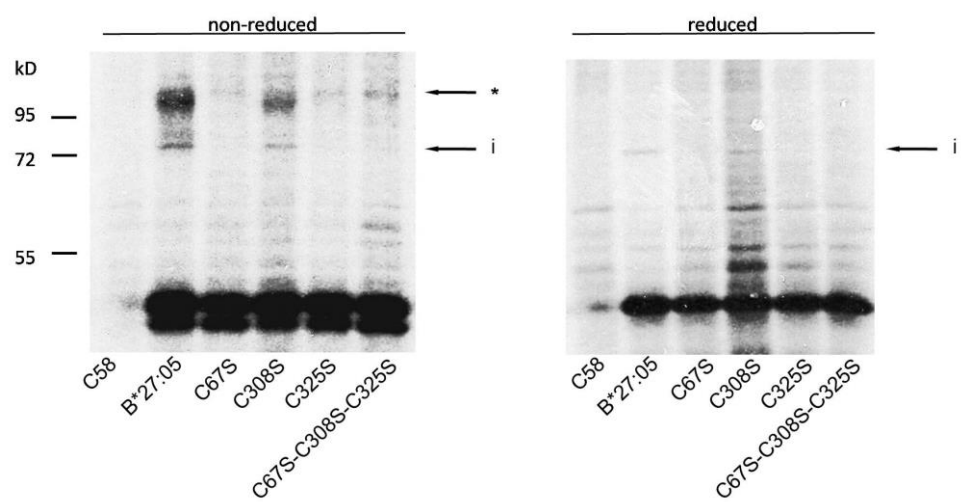


Figure 3.26. HLA-B27 ER resident dimers form at early and late MHC class I assembly stages. (A) C58 cells expressing HLA-B*27:05 wt, C67S, C308S, C325S and C67S-C308S-C325S mutants were pretreated with the alkylating agent NEM, lysed in 1% NP40 detergent and EndoH digested for 1hr at 37°C, prior to analysis by non-reducing SDS-PAGE and immunoblotting with HC10 that revealed EndoH-resistant (r) and -sensitive (s) heavy chain populations. (B) Cell lines from (A) were NEM pretreated and lysed in digitonin followed by immunoprecipitation with anti-TAP2 and analysed by non-reducing SDS-PAGE and immunoblotted with HC10 and ERp57 antibodies as a control. (C) Cells from (A) were metabolically labelled with [³⁵S]Met/Cys for 15 mins followed by immunoprecipitation with HC10 antibody. Immunoprecipitates were analysed non-reduced and reduced by SDS-PAGE. Arrow * indicates B27 dimer species whilst arrow 'i' indicates a band that is resistant to reduction and based on the Mw most likely represents co-precipitating BiP.

Additionally, since the unpaired cysteine at p308 has been proposed to participate directly in disulphide bonding between HLA-B alleles and the PLC (Gruda et al., 2007) (Chambers et al., 2008) the role of all three unpaired cysteines regarding association with the PLC was examined. Initially, cells were pretreated with NEM. To preserve hydrophobic interactions which can occur between components of the PLC, C58 cell lines expressing B27 wt, C67S, C325S, C308S and C67S.C308S.C325S were lysed in the presence of a mild non-ionic detergent digitonin, prior to immunoprecipitation with anti-TAP-2 antisera. Immunoblotting with HC10 revealed that HLA-B27 wt dimers were present within the PLC (Figure 3.26B, upper panel, lane 6). Moreover, mutation of the unpaired cysteine residues did not influence detection of homodimers within the PLC (Figure 3.26B, upper panel, lanes 3-6). Although less intensive, a homodimer band can be observed for B27.C308S mutant. The low levels of heavy chain dimers detected with the C308S mutant is most likely due to variation in cell numbers as indicated by the reduced level of ERp57 detected in the control immunoblots of the lysates (Figure 3.26B, bottom panel). Also, the HLA-B*27:05 mutant bearing substitutions of all three unpaired cysteines, including that at p308, dimerises within the PLC with an efficiency similar to the other mutants (Figure 3.26B, upper panel, lane 6). Taken together the above data indicate that the majority of detected HLA-B27 dimers are ER resident and that these dimers have the ability to associate with the PLC.

However, it has been previously demonstrated that ER-resident HLA-B27 unfolded dimer species can form at the early stage of heavy chain folding (Dangoria et al., 2002). To determine whether I can also detect this dimer

population, C58 cells expressing HLA-B27wt and B27- unpaired cysteine mutants were metabolically labelled with [³⁵S]Met/Cys for 15 mins. Immunoprecipitation of unfolded heavy chain with HC10 confirmed that HLA-B27 dimers can also be detected at the early assembly stage, soon after HLA-B27 synthesis (Figure 3.26C, left panel arrow *).

3.3.2.2 Characterisation of HLA-B27 Single Chain Trimer

HLA-B27 dimers accumulate in the absence of tapasin and β 2m (Dangoria et al., 2002), suggesting a possible role for the antigen presentation pathway and β 2m-association in dimer formation. Optimal peptide binding is crucial for the stability of HLA heavy chains and its final conformation (Springer et al., 1998) (Smith et al., 1993) (Yu et al., 1999). Moreover, MHC class I dimerisation was proposed to occur as a consequence of loss or unavailability of β 2m (Capps et al., 1993).

Since availability and affinity of peptide and β 2m influence each other, I wanted to determine their role in HLA-B27 dimerisation. Unlike most studies which utilise various cells lines deficient in certain components of the antigen presentation pathway, Daudi cells (β 2m^{-/-}), T2 cells (TAP^{-/-}) or 220 (Tapasin^{-/-}) cells, my experimental approach was based on supplying β 2m and peptide during HLA-B*27:05 heavy chain folding by physical association of these three components creating a Single Chain Trimeric (SCT) molecule (Figure 3.27A). Such SCTs have been reported to assemble efficiently and maintain covalent structure as determined by its recognition with mAb that only recognise correctly folded molecules such as W6/32 or BB7.2 (Yu et al., 2002; and Dr Darren

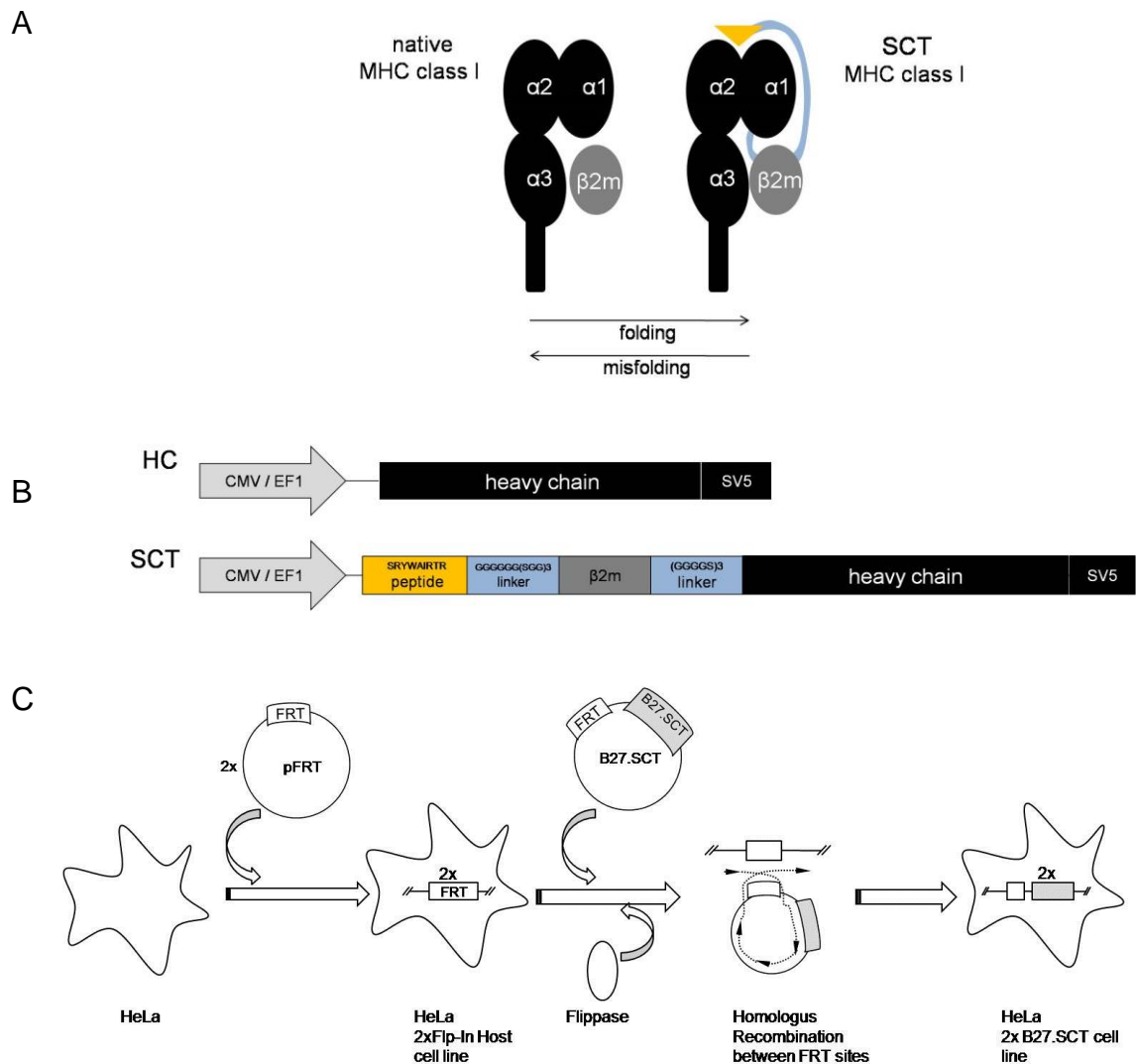


Figure 3.27. Structure and design of Single Chain Trimeric (SCT) molecules. (A) Schematic structure of the conventional MHC class I and SCT molecules. (B) Outline of the Heavy Chain (HC) and SCT constructs being composed of peptide- β 2m- V5-tagged heavy chain tethered together by a flexible linkers. (C) Outline illustrating generation of isogenic HeLa cell lines stably expressing 2 copies of SCT or HC constructs using a system based on Flp recombinase-mediated integration. HeLa cells were transfected with the Flp Recombination Target (FRT) site plasmid vector (pFRT) and the clone possessing two integrated α copies of the plasmid was selected. HeLa cells were then co-transfected with FRT-containing expression plasmid into which the HLA class I construct (B27 or B35) was cloned and with the plasmid expressing the Flp recombinase (Flippase). The homologous recombination between FRT sites facilitated by flippase resulted in integration of the gene of interest (B27.SCT/HC or B35.SCT/HC) at both target integration sites into the HeLa cells genome.

Nesmith, data not shown) and retain the attached peptide (Lybarger et al., 2003). Usage of SCT would allow me to determine the direct influence of peptide on HLA-B27 dimer formation. SCTs give us the opportunity to examine the influence of intrinsic properties of HLA-B*27:05 separately from other factors like assembly and peptide binding, as SCT have been proposed to be independent of ER chaperones and other accessory molecules (Kotsiou et al., 2011) (Primeau et al., 2005).

The SCT molecules were generated and kindly provided by Dr Keith Gould, Imperial College London. Briefly, the SCT comprises of the peptide sequence SRYWAIRTR (residues 383–391 of the influenza A virus nucleoprotein peptide [NP]) and the human β 2m amino-terminal hydrophobic signal sequence joined by a GGGGGG(SGG)₃ linker, a second (GGGGS)₃ linker, and V5 C-terminally tagged B*27:05 class I heavy-chain sequence (Figure 3.27B). The NP epitope was chosen for its predominant role in triggering an effective HLA-B27 restricted anti-influenza CTL response (Huet et al., 1990). The V5 tag facilitated allele-specific and efficient detection and isolation of constructs during the immunoblotting and immunoprecipitation procedures. For comparison with the SCT, a similar construct composed of Heavy Chain (HC) only was also generated. As an allelic control, non AS-associated HLA-B35 SCT and HC constructs were created in the same way. All the B27 and B35 constructs were expressed either under the strong Cytomegalovirus (CMV) or weak Elongation Factor 1 α (EF1) promoters (Figure 3.27B). The FLP-In system based on Flp recombinase-mediated integration was used to generate isogenic HeLa cell lines stably expressing physiological two copy numbers of both the B27 and

B35 HC/ SCT molecules (Figure 3.27C) (See Materials and Methods for details).

I initially wanted to test the expression and the function of our HLA-B27 SCT constructs. HeLa cell lines were FACS analysed with ME1 antibody (Figure 3.28). ME1 antibody staining revealed that both constructs were efficiently expressed at the cell surface (Figure 3.28A).

To determine whether these constructs could be efficiently recognized by CTLs, an HLA-B27 specific influenza NP T cell line was generated and the flow cytometric assays for monitoring cell-mediated cytotoxicity was performed. HeLa target cell lines expressing HLA-B27.HC alone as well as those expressing B27.SCT presenting NP peptide were un-pulsed or pulsed with NP-peptide and incubated with a NP-specific HLA-B27 restricted CD8⁺ CTL line. The CD8⁺ CTLs activation was measured by upregulation of CD107a also known as Lysosomal-associated membrane protein 1 (LAMP1) a transmembrane protein that localizes to lytic granules which are exocytosed during interaction with target cells (Peters et al., 1991). As shown in Figure 3.28 HeLa cells expressing B27.SCT with the covalently attached NP peptide are able to activate effector CTL cells whereas HeLa cells expressing B27.HC can only activate CTLs upon incubation with NP peptide (Figure 3.28). Hence recognition by the conformational-specific mAb ME1 and successful functional recognition by CTL confirms authentic assembly of HLA-B27 SCT constructs.

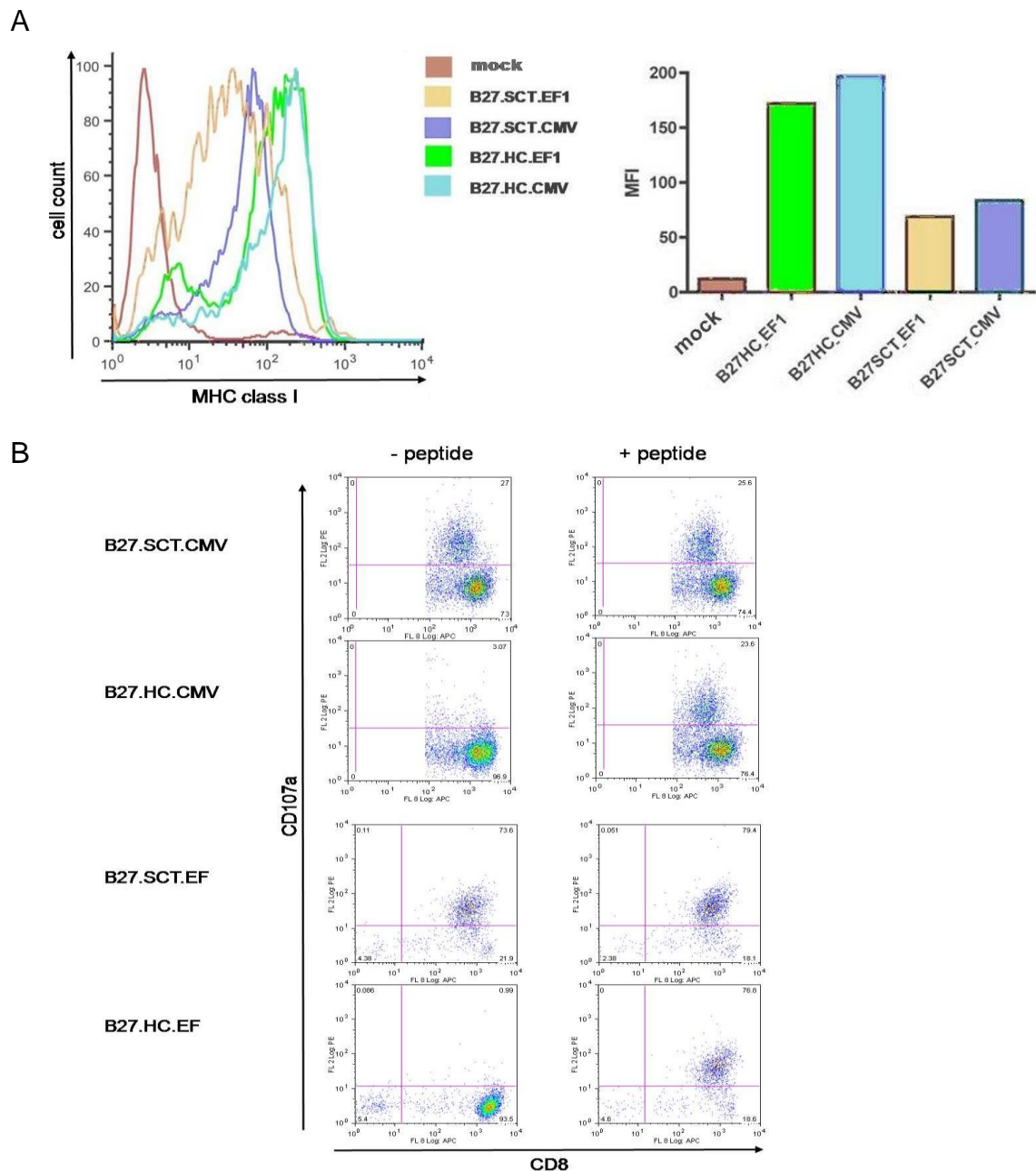
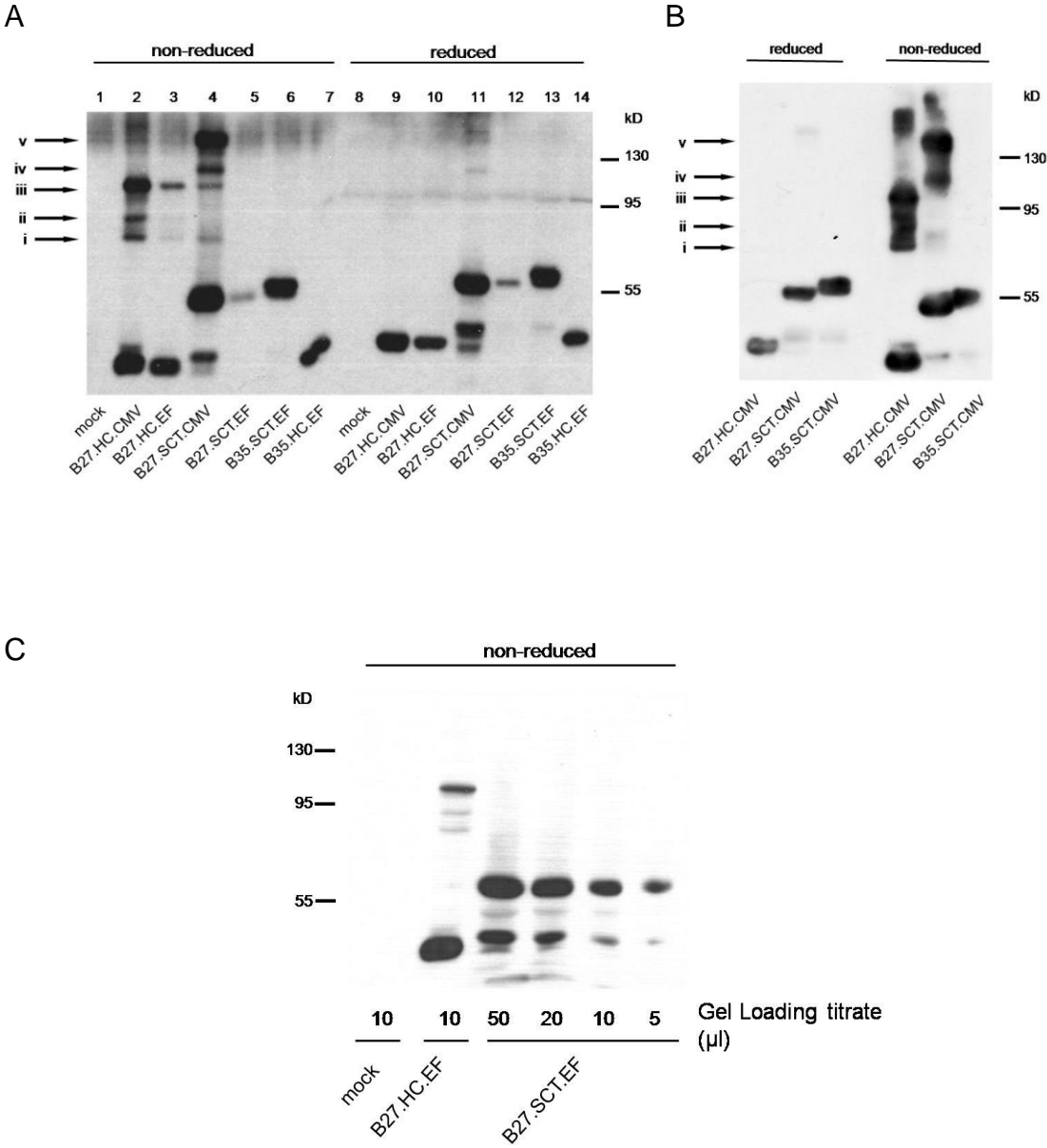


Figure 3.28. HLA-B27 SCTs are expressed at the cell surface and are functional. (A) HeLa cells expressing B27.SCT and B27.HC under eIF1 α (EF) or CMV promoter were stained with the conformational ME1 antibody followed by FACS analysis and presented as mean fluorescent intensity (MFI) (right panel). (B) Cells from (A) were unpulsed or pulsed with NP-peptide and incubated with an NP peptide-specific HLA-B27 restricted CD8⁺ CTL line and an anti-CD107a-APC antibody for 4h before further staining with CD8-APC and analysis by flow cytometry. Data is representative of 3 independent experiments: 2 carried out with an Effector to Target cells (E:T) ratio of 0.5:1 and 1 at an E:T ratio of 1:1.

3.3.2.3 HLA-B27 SCT dimerises and SCT Retains A Slow Maturation Phenotype

As HLA-B27 is prone to misfolding I wanted to test whether the SCT format could overcome the folding defects associated with HLA-B27. Therefore HLA-B27.SCT constructs were analysed at the protein level under both reducing and non-reducing conditions to determine whether the SCT exhibited any degree of misfolding. HeLa cells expressing the given constructs were pretreated with the alkylating agent NEM, followed by lysis in 1% NP40 detergent. Immunoblotting with the anti-V5 epitope tag antibody pK of lysates revealed that HLA-B27.HC can form disulphide linked dimers (Figure 3.29A, lanes 2 and 3, arrows i-iii). Surprisingly, HLA-B27.SCT.CMV was also capable of dimerisation (Figure 3.29A, lane 4, arrows iv-v). On the other hand, B27.SCT.EF, unlike the corresponding construct under the stronger CMV promoter, did not dimerise (Figure 3.29A, compare lane 4 and 5). Nevertheless, dimerisation of the B27.SCT.CMV molecule cannot be simply accounted for overexpression, as HLA-B35, a non AS-associated allele, under the same strong promoter did not dimerise (Figure 3.29B). Moreover B35, did not form dimers neither as an HC or SCT (Figure 3.29A, lanes 6 and 7).

Although, equal numbers of each cell line were used in this experiment, the lack of a B27.SCT.EF dimer band could be the result of the low B27 protein levels. Therefore, I titrated the amount of lysate generated from cells expressing B27.SCT.EF and B27.HC.EF. Immunoblotting of increasing amounts of lysate did not reveal any dimers, thus confirming that B27.SCT under the weaker EF1 promoter does not dimerise (Figure 3.29C). These results emphasize the



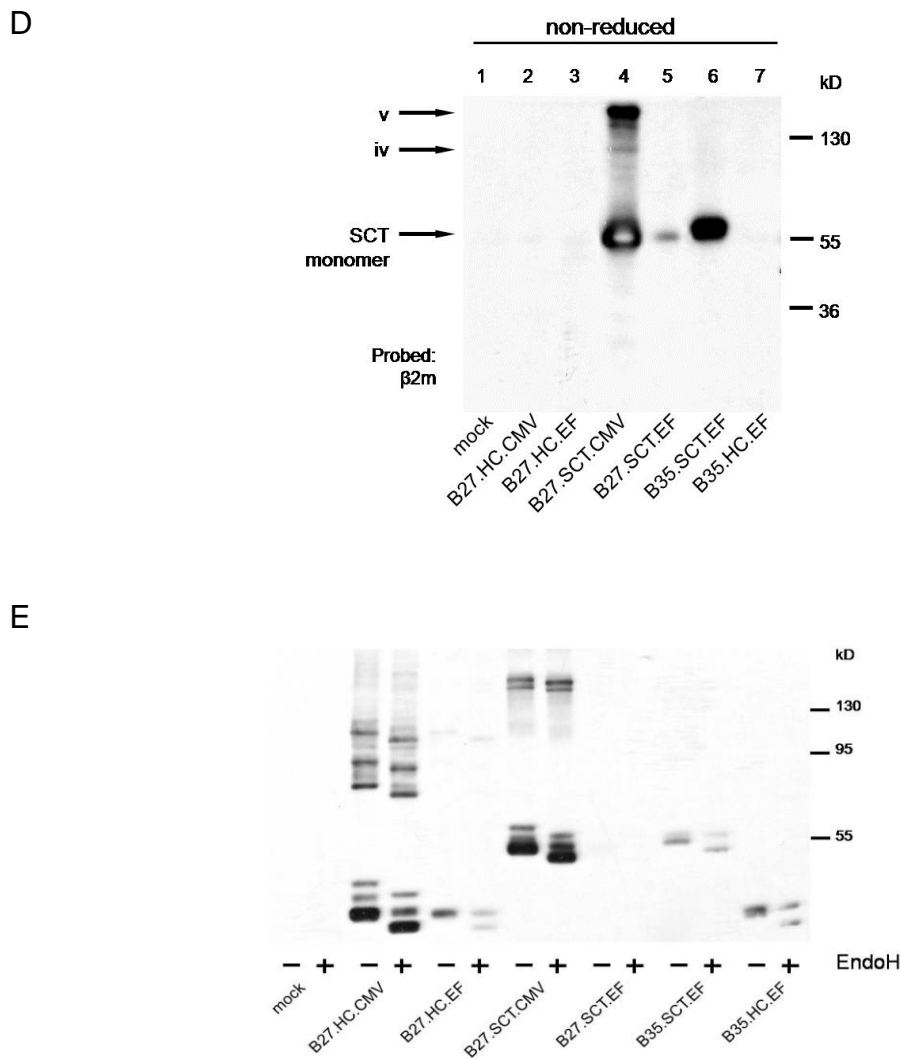


Figure 3.29. HLA-B27 SCT forms disulphide bonded dimers within the ER. (A-B) NEM-treated lysates from HeLa cells mock transfected and expressing B27.HC.CMV/EF, B27.SCT.CMV/EF, B35.HC.EF and B35.SCT.EF/CMV were analyzed by SDS-PAGE, followed by immunoblotting with pK antibody. **(C)** NEM-treated lysates of B27.SCT.EF was titrated and separated by non-reducing SDS-PAGE and compared against B27.HC.EF. **(D)** Lysates from (A) were immunoblotted with anti- β 2m antibody. **(E)** Lysates from (A) were EndoH digested prior to immunoblotting with pK antibody. Arrows i-v indicate high molecular HC and SCT species.

uniqueness of HLA-B27 to form heavy chain dimers and indicate that fully folded B27 in the form of SCT retains the ability to dimerise. These data also confirm that HLA-B27 upregulation can correlate with the enhanced heavy chain dimerisation (Tran et al., 2004) (Taurog et al., 1993).

To exclude whether the B27.SCT high molecular species (Figure 3.29A, lane 4, arrows iv-v) did not arise from molecules that had lost β 2m, possibly due to degradation events, the filter was stripped and reprobed for β 2m (Figure 3.29D). Immunoblotting confirmed the presence of β 2m within the majority of B27.SCT dimers (Figure 3.29D, arrows iv and v). However, a small population of B27.SCT molecules seems to decay and run at Mw similar to the B27.HC dimer band (Figure 3.29A, compare lanes 2 and 3 with 4, arrows iii and i). Also the B27.SCT monomer seems to undergo some degree of proteolytic digestion which generates bands of similar Mw to the B27.HC (Figure 3.29A, lane 4). Immunoblotting for β 2m suggested that the degradation product arose from the B27.SCT complex losing the β 2m subunit (Figure 3.29D, arrows iv and v). Nevertheless, since β 2m can be detected in the B27.SCT dimers (Figure 3.29D, arrows iii and iv) it indicates that although some proteolytic degradation of the SCT complexes takes place, the B27.SCT detected dimers occur between two B27.SCT molecules that maintain an intact covalent interaction with β 2m.

To determine the localisation of B27.HC and B27.SCT dimers, lysates were digested with Endo H to determine if the appropriate sugar modifications associated with the ER to Golgi transit were present. Following incubation with EndoH, it appeared that the majority of both HLA-B27.HC and HLA-B27.SCT dimers were ER resident (Figure 3.29E).

We have previously reported that HLA-B27 dimer formation within the ER was associated with slow folding kinetics (Antoniou et al., 2004). However it has been postulated that MHC class I SCTs have a rapid pulse chase phenotype (Primeau et al., 2005). Thus to determine whether the correlation between the slow folding and dimer formation was applicable to HLA-B27.SCT I analysed the maturation rate of HLA-B27.SCT in comparison to HLA-B27.HC and B35 using pulse chase analysis. HeLa cells expressing B27.HC, B27.SCT and B35.HC were metabolically labelled with [³⁵S]Met/Cys, chased for 0, 1, and 2 hours, immunoprecipitated using the anti-epitope V5 tag antibody (pK), followed by digestion with Endo H and SDS-PAGE analysis. Surprisingly, the analysis revealed that HLA-B27.SCT had a similar slow pulse chase phenotype to the HLA-B27.HC since the majority of both B27.HC and B27.SCT remain EndoH sensitive at 0, 1h and 2h of time point (Figure 3.30 A and B, left panels). On the contrary, the HLA-B35 allele revealed much more rapid maturation kinetics, since at the first time points around 2 times more B35 protein becomes EndoH resistant in comparison with B27.HC and B27.SCT (Figure 3.30B, left panels).

Within 2 hours of chase, the majority of the B27 population appeared to remain within the ER compartment (SCT or HC) (Figure 3.30 A and B, left panels). To determine the maturation kinetics for B27.SCT and HC, a similar experiment was performed with extended times of chase for up to 8 hours (Figure 3.30A, right panels). This pulse chase analysis revealed that both B27.HC and B27.SCT possess slow folding kinetics as even after 8 hours more than 50% of the molecules are still Endo H sensitive (Figure 3.30B, right panels). However,

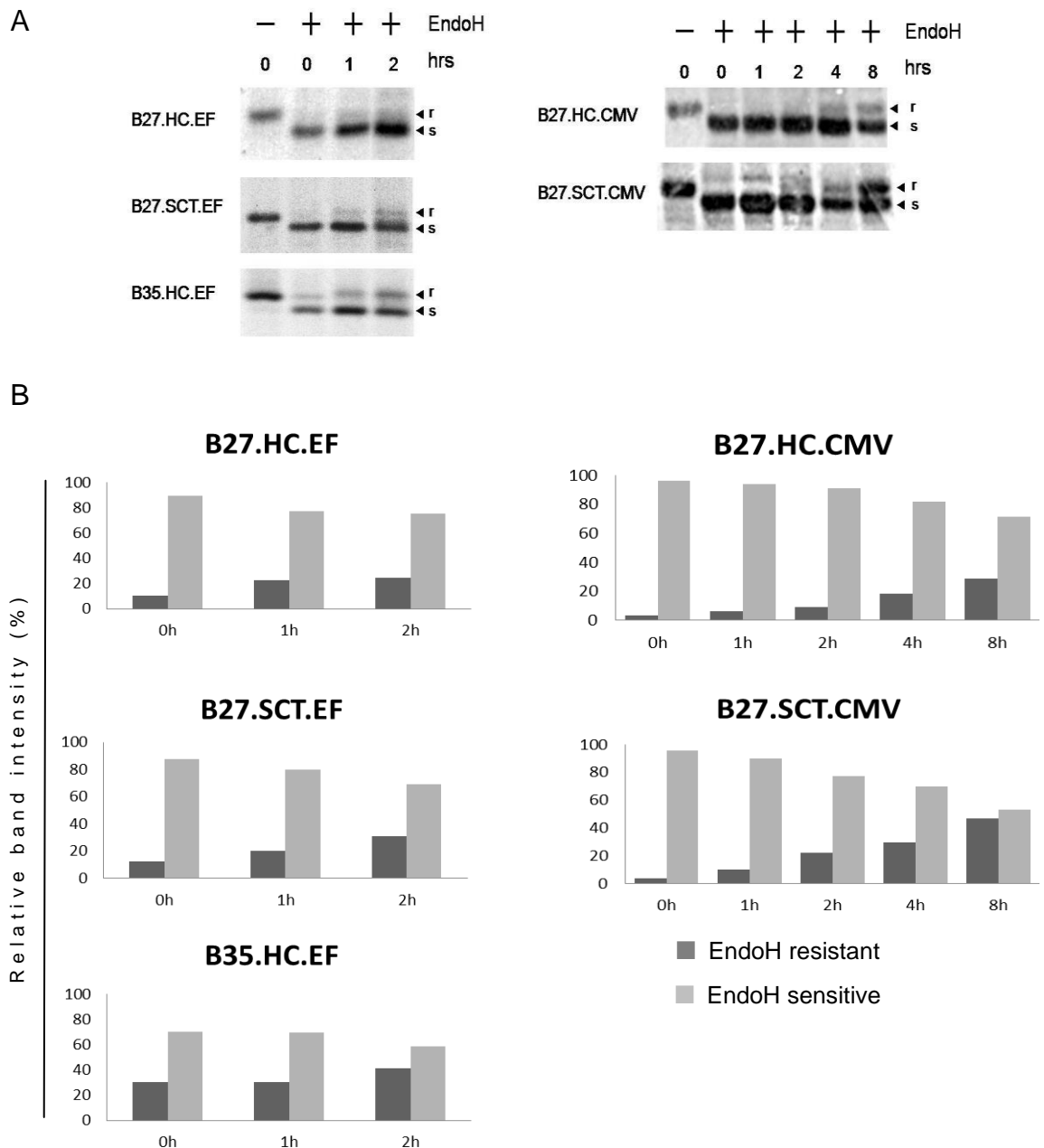


Figure 3.30. HLA-B27 SCT exhibits slow folding kinetics. (A) HeLa cells mock transfected and expressing B27.HC.CMV/EF, B27.SCT.CMV/EF and B35.HC.EF were metabolically labeled and chased for 0, 1, and 2 hrs (left panel) or up to 8hrs (right panel) followed by immunoprecipitation with pK antibody. Immunoprecipitates were EndoH-digested and analyzed by reducing SDS-PAGE. Arrows r and s indicate EndoH-resistant and –sensitive heavy chains, respectively. (B) The percentage of the radioactivity detected in the Endo-H–sensitive or Endo-H–resistant bands was plotted as the proportion relative to the total radioactivity obtained from the Endo-H–sensitive and –resistant species.

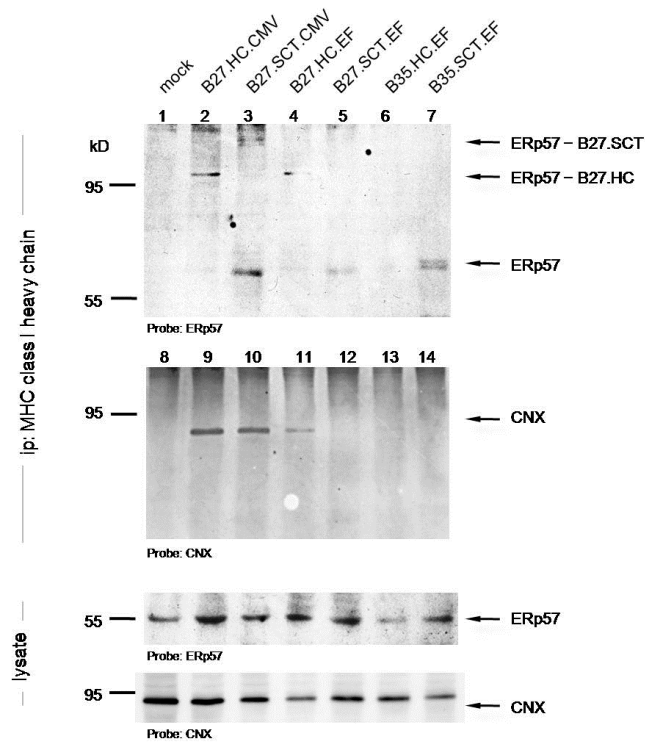
at the latest time point B27.HC has more EndoH sensitive material in comparison with SCT indicating that the overall SCT egress from the ER is more efficient than B27 not combined with peptide and protein (Figure 3.308, right panels). Taken together, these results revealed that the SCT format cannot completely rectify potential folding problems associated with MHC molecules such as HLA-B27. Furthermore, this analysis highlights that HLA-B27, regardless of the physical linkage with $\beta 2m$ and peptide possesses an inherent ability to fold slowly, which would account for its ability to dimerise.

3.3.2.4 MHC Class I SCT molecules Can Associate With The ER Resident Chaperones

There is little known about the cellular processing events and biochemical properties of SCTs. Association of SCT with other ER chaperones, except TAP and tapasin (Primeau et al., 2005) i.e. calreticulin, calnexin, ERp57 and PDI, have not been examined. Our group has demonstrated that HLA-B27 molecules susceptible to dimerisation show prolonged and strong associations with ER resident chaperones (Antoniou et al., 2004). Therefore, I wanted to determine whether chaperones do indeed associate with HLA-B27 in the form of an SCT molecule.

To analyse the association of chaperones during the early and latter stages of folding the HeLa isogenic cell lines expressing given HC and SCT constructs were pretreated cells with NEM followed by immunoprecipitation of the MHC class I heavy chain under harsh detergent conditions. Immunoblotting of immunoprecipitates revealed that both ERp57 and CNX could be co-precipitated with both the HLA-B27.HC and SCT. ERp57 immunoblotting

A



B

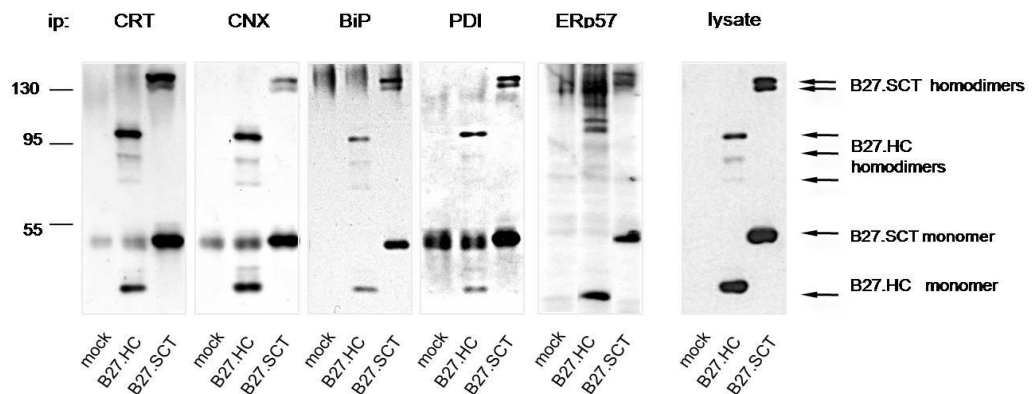


Figure 3.31. HLA-B27 in the form of an SCT demonstrates associations with ER folding chaperones. (A) HeLa cells mock transfected, B27.HC.CMV/EF, B27.SCT.CMV/EF and B35.HC/SCT.EF were treated with NEM, lysed, and immunoprecipitated with pK antibody. Immunoprecipitates were resolved by non-reducing SDS-PAGE and analyzed by immunoblotting with ERp57 and calnexin (CNX) antibodies. (B) Cells expressing B27.HC and SCT were pretreated with NEM, lysed and immunoprecipitated with carleticulin (CRT), CNX, Immunoglobulin binding Protein (BiP), Protein disulphide isomerase (PDI) and ERp57, followed by immunoblotting with pK antibody for detection of MHC class I heavy chain.

revealed a differential pattern of association (Figure 3.31A). ERp57 could be detected in direct association with HLA-B27.HC (Figure 3.31A, upper panel, lane 2 and 4) as previously reported (Antoniou et al., 2002) (Santos et al., 2007), but predominantly as a monomer, in immunoprecipitates of the SCT (Figure 3.31A, upper panel, lane 3 and 5). Calnexin, on the other hand, was only detected in immunoprecipitates from cell lines expressing B27.HC.CMV/EF and B27.SCT.CMV i.e. only molecules which were found to dimerise (Figure 3.31A, lane 9, 10 and 11).

The above experiment does not reveal whether monomeric or dimeric MHC class I structures associate with the given chaperones. To determine whether monomeric or dimeric forms of HLA-B27 HC or SCT associated with chaperones, the respective cell lines were NEM treated, lysed and immunoprecipitated for CNX and ERp57 as well as CRT, BiP and PDI. Immunoblotting for HLA molecules revealed that both heavy chain monomers as well as dimer species of either B27.HC and B27.SCT can be co-precipitated with CRT, CNX, BiP, PDI and ERp57 (Figure 3.31B).

This is the first demonstration that MHC class I SCT molecules can interact with ER resident chaperones, strongly suggesting that despite the presence of peptide and β 2m, there are intrinsic properties of the B27 heavy chain which determine requirement for ER chaperone assistance.

3.3.2.5 HLA-B27 forms heavy chain and SCT homodimers within the PLC

The above analysis though only examined chaperone interactions during the early stages of SCT biosynthesis. The later stages of MHC class I molecule folding are characterized by the formation of the PLC. The presence of peptide

would presumably allow the SCT complex to omit the PLC stage of assembly when the exogenous peptide is loaded onto the canonical MHC class I heavy chain. To determine if covalent attachment of the NP peptide in the B27.SCT complex allows the molecule to circumvent the PLC, cell lines were pretreated with NEM, prior to lysis in the non-ionic detergent digitonin to help preserve the hydrophobic interactions between members of the PLC. Lysates were immunoprecipitated either with anti-TAP or -tapasin antibodies to examine whether HLA-B27 molecules in the form of HC or SCT can associate with PLC. Immunoblotting revealed that HLA-B27.SCT does indeed associate with the PLC as a monomer (Figure 3.32A). Surprisingly, we could also detect HLA-B27.HC and SCT dimers within the PLC (Figure 3.32A, arrows i-iii and iv-v, respectively). This seems to be the first report of HLA class I dimerisation within the PLC. Over exposure of the film revealed that monomers of B35.SCT and B35.HC can be detected in association with both TAP and tapasin but at the lower levels than HLA-B27.

MHC class I heavy chain-tapasin conjugates have been proposed to occur via unpaired cysteine residues located within the cytoplasmic domain of their proteins in an *in vitro* translation system (Chambers et al., 2008). However, we found that mutating the unpaired C67, C308 and C325 did not abrogate the association of B27.SCT with the PLC (Figure 3.32B, arrow*) indicating that unpaired cysteines are not key residues responsible for the binding of the heavy chain to tapasin. Moreover, the presence of B27.SCT dimers, despite the substitution of unpaired cysteines (Figure 3.32B, arrow iv-v), further confirms

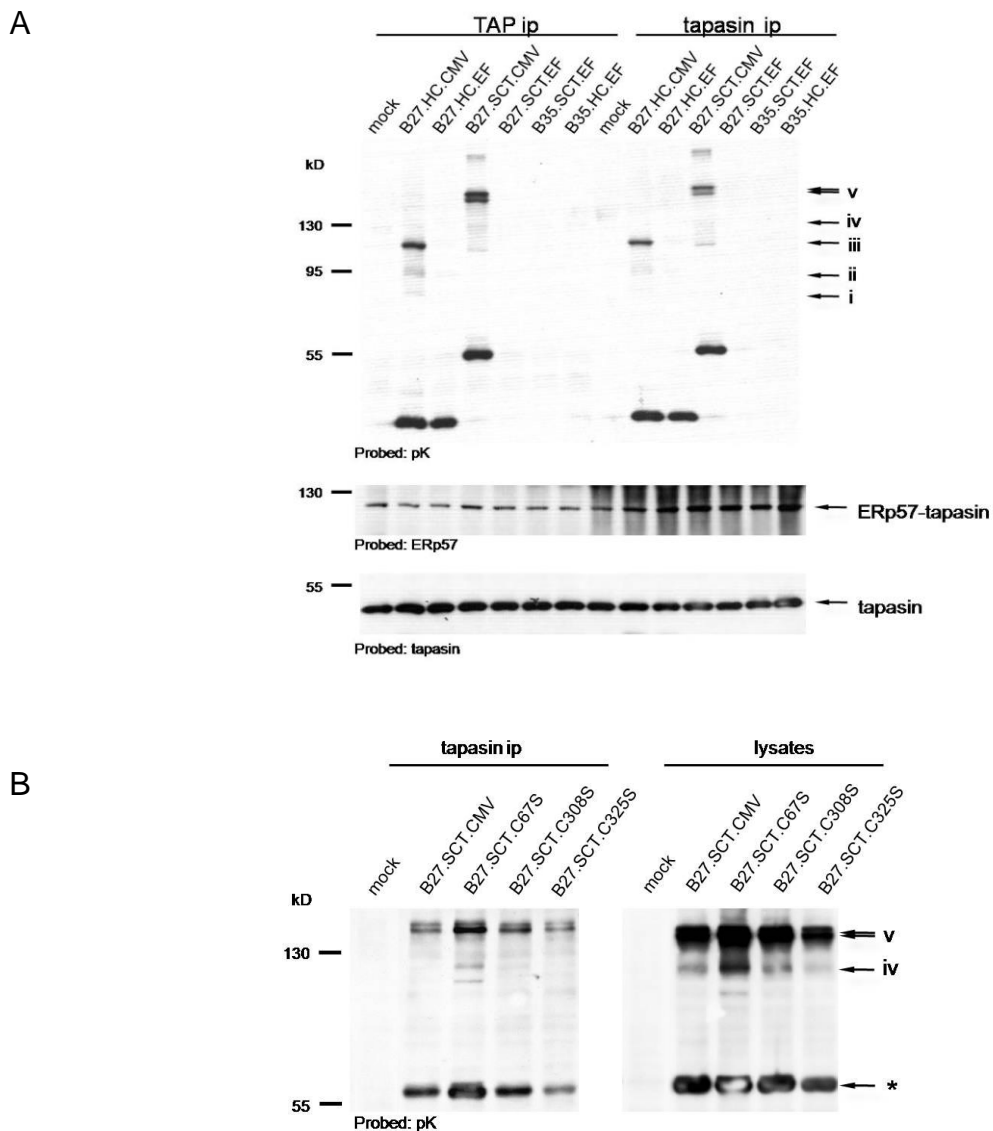


Figure 3.32. HLA-B27 SCT monomers and dimers associate with the PLC. (A) HeLa cells mock transfected and expressing B27.HC.CMV/EF, B27SCT.CMV/EF, B35.HC/SCT.EF were pretreated with NEM prior to lysis in 1% digitonin followed by immunoprecipitation with anti-TAP1 or –Tapasin antibodies. Immunoprecipitates were resolved by non-reducing SDS-PAGE and immunoblotted for MHC class I heavy chain with pK (top panel), ERp57 (middle panel) and Tapasin (bottom panel) Abs. (B) B27.SCT.CMV construct was mutated to generate B27.SCT.C67S, C308S and C325S mutants that were stably expressed in the HeLa FLP isogenic cell line. Following pretreatment with NEM, lysates were immunoprecipitated with an anti-tapasin antibody and separated by non-reducing SDS-PAGE, followed by immunoblotting with pK antibody. Arrows i-v indicate high molecular HC and SCT species respectively, and * - heavy chain monomer.

my earlier observations in chapter 1 that HLA-B27 dimer formation predominantly involves conserved cysteine residues. It is evident that HLA-B27.HC and SCT do indeed form high molecular weight dimers both within and without the PLC. I therefore wanted to determine whether these dimeric structures were indeed homodimers or heterodimers comprised of B27.SCT and a protein that has previously been described to form mixed disulphide conjugates with MHC class I heavy chains such as ERp57, PDI or tapasin (Santos et al., 2007) (Park et al., 2006) (Chambers et al., 2008). Hence, lysates and immunoprecipitates were probed for PDI, ERp57 and tapasin. However, none of these proteins could be detected within the dimeric complex (Figure 3.33A).

To determine the composition of the high Mw structures detected with the PLC more thoroughly, 2D isoelectric focusing gel electrophoresis was performed, where TAP immunoprecipitates were separated according to charge followed by Mw resolution (Figure 3.33B). I reasoned that if these dimers were composed of proteins other than two MHC class I molecules, the dimeric form of both HLA-B27.HC and SCT would run in the absence of a correlating monomer band. Two dimensional analysis revealed that the latter possibility was not the case and all B27.HC dimers (Figure 3.33B, arrows i-iii) and SCT dimers (Figure 3.33B, arrows i-ii) have corresponding monomer bands (Figure 3.33B, arrow *). Thus, both lysates (Figure 3.33A) and TAP immunoprecipitates (Figure 3.33B) confirmed that the high Mw structures represent true HLA-B27 homodimers and exclude other proteins.

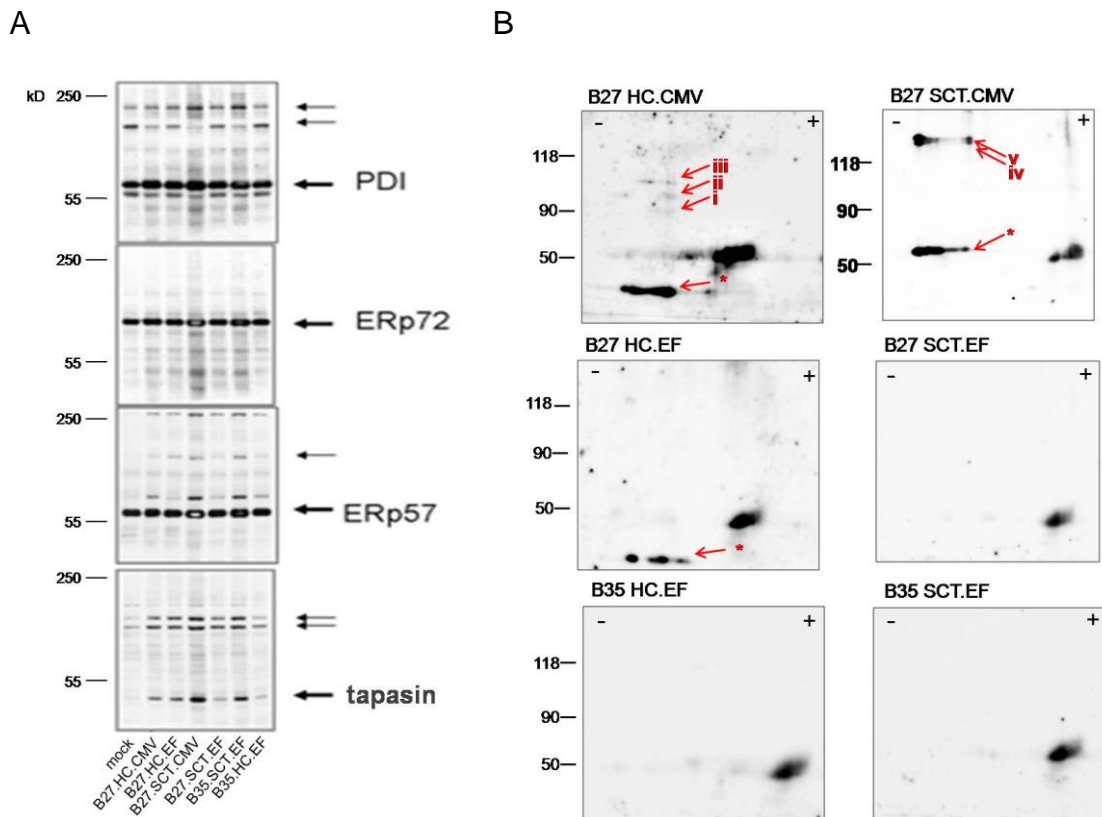


Figure 3.33. HLA-B27 HC and SCT high molecular species are composed of two B27 molecules. (A) HeLa cells expressing B27.HC and -SCT constructs were analyzed along with B35.HC, B35.SCT and HeLa mock control. Cell lines were NEM-treated, lysed in 1% NP40 and analyzed non-reduced by immunoblotting with PDI, ERp72, ERp57 and tapasin. Unlabelled arrows indicate high molecular bands revealed by immunoblotting which do not correlate with B27.HC and SCT homodimers bands detected previously. (B) TAP-immunoprecipitates of the cell lines from (A) were analysed non-reduced by a two-dimensional electrophoresis, followed by immunoblotting with pK antibody. Arrows indicates B27.HC (i-iii) and B27.SCT (iv-v) homodimers and monomers (*) detected in association with PLC.

3.3.2.6 Residue 116 influence HLA-B27 dimerisation

Detection of HLA-B27 within the PLC even in the form of an SCT which hypothetically should obviate the requirement for antigen processing, raises the possibility that the HLA-B27 PLC-dependency profile could correlate with its dimerisation phenotype. The MHC class I heavy chain association with the PLC has been reported to be dependent on residues at p114 and p116 of the MHC class I heavy chain which form part of the peptide binding F pocket (Williams et al., 2002). The HLA-B27 subtypes -06, -09 and -07, which demonstrated negative or weak associations with AS (Cauli et al., 2007) (Gomez et al., 2006) (Olivieri et al., 2007) (Ramos et al., 2002b) (Varnavidou-Nicolaidou et al., 2004) (Galocha and Lopez de Castro, 2010) display natural polymorphisms within this region (see Table 3). Moreover, AS-related and non-related subtypes were found to differ in their tapasin-dependency with respect to cell surface expression and PLC association (Goodall et al., 2006).

To determine if the association of the B27.SCT within the PLC can be influenced by 114 and 116 polymorphisms despite a covalent linkage of heavy chain to peptide, site directed mutagenesis was performed to create the B27.SCT.H114D-D116Y mutant, mimicking the HLA-B*27:06 subtype which was previously found not to associate with the PLC (Goodall et al., 2006). Tapasin immunoprecipitation experiments, performed as described above, revealed that monomers of B27.SCT.H114D-D116Y molecules can be co-precipitated with tapasin with similar efficiency as the B27.SCT wt molecule (Figure 3.34A, left panel, arrow *), whilst dimers of the F pocket mutant were not

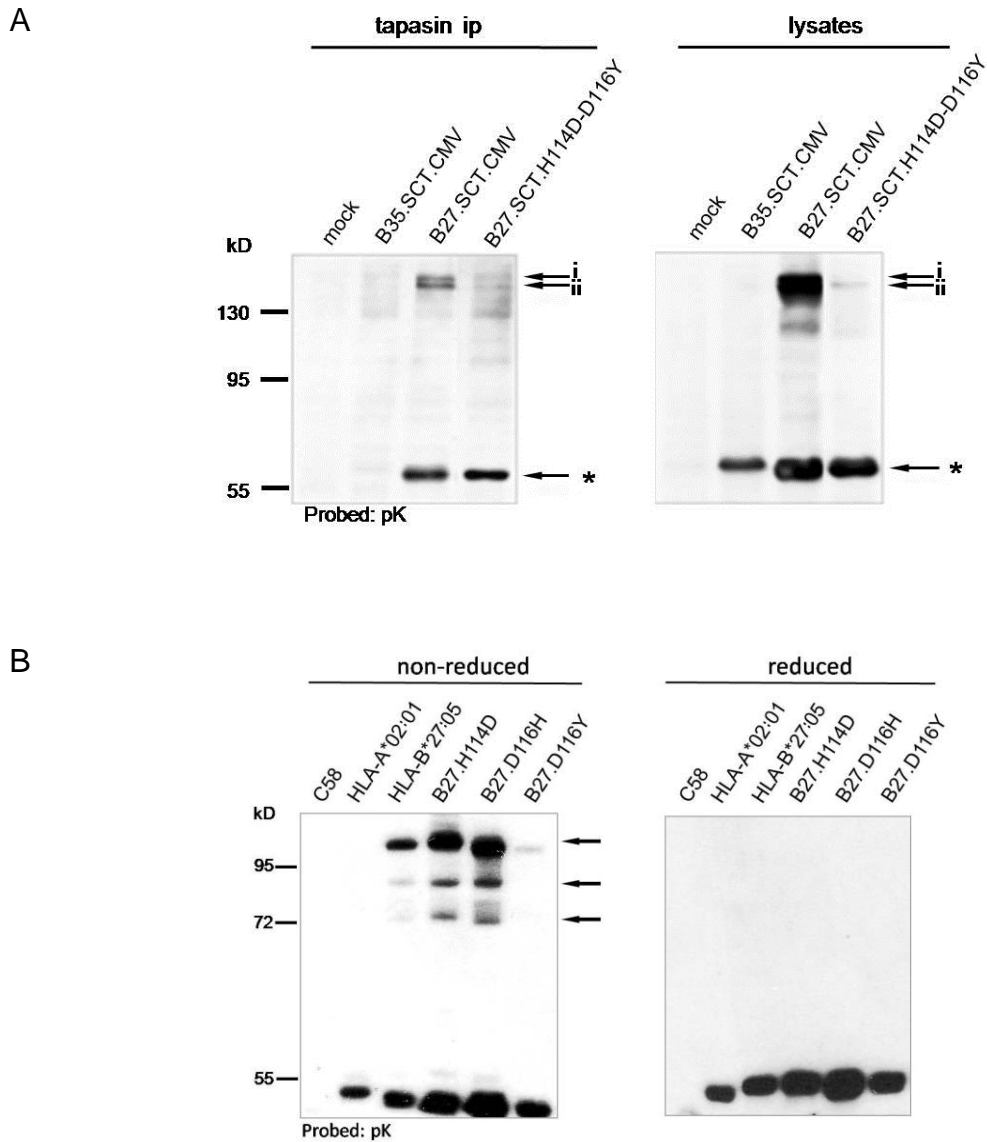


Figure 3.34. HLA-B27 homodimer formation is influenced by residues expressed at p116 within the F pocket of the peptide binding groove. (A) HeLa cells mock transfected and expressing B35.SCT.CMV, B27SCT.CMV and B27.H114D-116Y mutant, were pretreated with NEM prior to lysis in 1% digitonin followed by immunoprecipitation with anti-Tapasins antibody. Immunoprecipitates (left) and lysates (right) were separated by non-reducing SDS-PAGE and immunoblotted for MHC class I heavy chain with pK. **(B)** C58 cells expressing HLA-A*02:01 and -B*27:05 wild types, B27.H114D, B27.D116H and B27.D116Y were pretreated with NEM, lysed and analysed reduced and non-reduced by immunoblotting with pK. Arrows indicate detected B27 heavy chain homodimer species and arrow* heavy chain monomer.

detected. Immunoblotting of cell lysates revealed that mutation of residue 114 and 116 diminishes the overall SCT dimerisation drastically (Figure 3.34A, right panel), thus explaining the lack of B27.SCT.H114D-D116Y dimers in association with tapasin.

The above experiment identified residues 114 and 116 as important in B27 dimerisation. To further characterise whether one or both residues account for the altered dimerisation phenotype, residues 114 and 116 were mutated separately within an HLA-B*27:05 cDNA sequence. Additionally, the B27.D116H mutant, which mimics the HLA-B*27:09 allele, was generated. Interestingly, this is the only residue which distinguishes the B*27:05 and B*27:09 alleles and at the same time, it has been proposed to correlate with stronger associations of the 09 subtype with the PLC and minimal homodimer formation (Blanco-Gelaz et al., 2009). C58 cell lines stably expressing B27.H114D, B27.D116H, B27.D116Y, as well as HLA-B*27:05 and HLA-A*02:01 wild type molecules for reference, were pretreated with NEM, lysed in 1% NP40 detergent followed by immunoblotting with anti-V5 tag, pK antibody. This analysis revealed that that B27.H114D and B27.D116H mutants can dimerise with similar efficiency as wild type HLA-B*27:05 molecules (Figure 3.34B). Interestingly, the introduction of a tyrosine at p116 was the only substitution which diminished dimerisation drastically (Figure 3.34B).

Taken together, the data within this chapter indicate that the presence of β 2m and optimal peptide have the potential to improve the folding properties of HLA-B*27:05 heavy chain leading to diminished dimerisation. However, HLA-B27 is specifically prone to form ER resident disulphide bond homodimers both at the

early and late stages of assembly. Furthermore, homodimerisation can be promoted by high protein expression levels. I also confirmed the relationship between HLA-B27 dimer formation and the slow maturation rate. The apparent slow maturation kinetics was observed irrespective of the presence of β 2m and peptide. Furthermore, dimerisation strongly correlated with heavy chain association with ERp57 and CNX. The HLA-B*27:05 dimerisation phenotype, can be reversed by a single substitution of aspartic acid at 116 to tyrosine, which is naturally shared by two weakly/ non-AS associated B*27:06 and B*27:07 subtypes. Furthermore, by substituting H114DD116Y in the SCT also lead to a pronounced decrease in dimers that were or were not associated with the PLC.

3.3.3 Discussion

HLA-B27 dimerisation has been detected by myself, previously by our laboratory and others, however the mechanism underlying this process remains undetermined. The folding of MHC class I molecules is predominantly determine by peptide loading and interactions with the PLC. The same HLA molecule can adopt several different conformations according to its peptide cargo. These latter observations suggested that HLA-B27 dimer formation could be influenced by the peptide and/or the components of the PLC. However, no previous experiments have been performed to demonstrate this. One of the major findings of this chapter was the demonstration that HLA-B27 dimers could be found within the PLC. However, the presence of high affinity peptide and β 2m did not rectify potential folding problems associated with HLA-B27

manifested by the slow folding kinetics, the association with ERp57 and CNX chaperones and ultimately, aberrant heavy chain dimer formation. Strikingly, my study demonstrated that point mutations of the residue at p116 within the F pocket of the peptide binding groove, has a dramatic impact on HLA-B27 dimerisation.

The presence of B27 dimers within the PLC was unexpected, as the CNX/CRT quality control system would be assumed not to allow defective molecules to proceed further along the assembly pathway. MHC class I incorporation into the PLC is determined by the heavy chain association with β 2m (Santos et al., 2007). If so, the PLC-associated dimers could form within the PLC from B27 heavy chains incorporated into the PLC as two β 2m-associated monomers. Alternatively, β 2m-associated dimers could form prior to PLC formation and could be recruited into the PLC as already disulphide linked heavy chain dimers. This latter scenario assumes that folded, β 2m-associated dimers would be relatively abundant within the ER, since they would form prior to PLC incorporation. Although this hypothesis cannot be excluded, studies by Dangoria and colleagues argue against this since they reported that folded (W6/32-reactive) B27 dimers appear mainly at the cell surface whereas ER-resident dimers consist of unfolded dimer species (Dangoria et al., 2002). Hence in light of the data presented here and previous studies it is more likely that HLA-B27 dimers detected within the PLC originate from two molecules incorporated into the PLC as heavy chain, β 2m-associated monomers and their coupling within the PLC could be accompanied by the loss of β 2m. However, my immunoprecipitation experiment does not discriminate whether detected

PLC-associated dimers are β 2m-free or associated. Such a scenario could be tested by TAP immunoprecipitation followed by reimmunoprecipitation with HC10 and ME1 antibodies respectively.

HLA-B27 dimers were also found to form rapidly after heavy chain synthesis (Dangoria et al., 2002). Notably this dimer population, consisting mainly of the “unfolded”, β 2m-free two heavy chains (Dangoria et al., 2002), were also detected by us. The unfolded, HC10-reactive high Mw HLA-B27 species were observed within 15 min of radiolabeling. The formation of dimers at both “early” and MHC class I late assembly stage does not exclude each other. Furthermore, it seems that these two populations may form independently of each other. The unfolded dimers that form rapidly after heavy chain synthesis may not proceed further in the assembly process due to the absence of β 2m. Such dimers could be considered as unfolded molecules and therefore be degraded by the ERAD system. On the contrary, dimers that form within the PLC could be more prominent and long lived as previous observations in the TAP-deficient cell line could not detect neither W6/32- nor HC10-reactive dimers (Dangoria et al., 2002).

Most studies that have examined the role of peptide and PLC in HLA-B27 misfolding and dimerisation have relied on cells lacking one or more assembly pathway components such as cell lines deficient in β 2m (e.g. Daudi cells), peptide transporters (e.g. T2 cells), or tapasin (e.g..220 cells). However, since these components of the MHC class I assembly pathway act in a co-operative manner the absence of one component could have direct and indirect effects which are hard to distinguish. Therefore it is difficult to discriminate whether

effects are due to the alteration in PLC assembly (even those beyond detection) and/or perturbation in peptide loading. In this regard, I took advantage of the SCT, the MHC class I heavy chain have been covalently attached to $\beta 2m$ and peptide. Despite the SCT being designed to improve the efficiency of MHC class I assembly, it did not rectify potential folding problems associated with HLA-B27 nor prevent heavy chain dimer formation.

I confirmed that protein overexpression enhances the ability of HLA-B27 to form heavy chain dimers (Taurog et al., 1993) as B27 expressed under a strong CMV promoter continued to form dimers despite being physically linked to the $\beta 2m$ and high affinity peptide. However, although MHC class I upregulation was shown to induce dimerisation even in non-AS associated alleles, my results also indicated that it cannot be generalized that protein overexpression broadly leads to dimerisation since HLA-B35 did not demonstrate aggregate formation even under the control of a strong promoter. Conversely, my results demonstrate that dimerisation is not simply the results of protein overexpression since despite only two copies of HLA-B27 molecule being expressed in HeLa cell line, B27 retains the tendency to dimerise. Hence there are some intrinsic properties of the HLA-B*27:05 molecule that predispose it to dimerisation. This intrinsic property unlikely can be accounted for the number of cysteine residues since HLA-B27 has only two more cysteine residues in comparison with HLA-B35.

My results extend previous findings that noted HLA-B*27:05 dimerisation strongly correlated with the slow maturation rate (Antoniou et al., 2004). Here I noted that HLA-B27 continues to fold slowly despite being physically linked to high affinity peptide and $\beta 2m$. The MHC class I assembly stage which largely

influences the export rate of the heavy chain from the ER, is the association with the PLC. The MHC class alleles that poorly associate with the PLC were shown to possess the faster assembly rate in comparison with the heavy chain that were incorporated into the PLC (Neisig et al., 1996) (Blanco-Gelaz et al., 2009) (Goodall et al., 2006). In accordance with these observations I demonstrated that both native HLA-B27 as well as B27.SCT fold slowly and associate with the PLC. On the contrary HLA-B35, a non-disease associated allele, which exhibits a faster folding rate, reveals little or no association with the PLC and does not form heavy chain dimers.

It is intriguing that the B27.SCT, despite the presence of high affinity peptide is incorporated into the PLC similarly to native HLA-B27. Peptide optimisation is the intrinsic property of the MHC class I molecule that in turn is strongly dependent on the structure of the peptide binding groove which is influenced by residues comprising the peptide binding cleft. Particularly residues 114-116 located within F pocket affect whether peptide optimisation occurs in a tapasin-dependent manner (Williams et al., 2002). Natural polymorphisms exist between AS-associated and non/weakly AS-associated B*27:09, B*27:07 and B*27:06 subtypes. I found that residue 116 has a profound influence on B27 folding and dimer formation. The D116Y mutation reduced HLA-B27 dimerisation even in a protein overexpression system. Introduction of a tyrosine at p116, which is shared by B*27:06 and 07 subtypes, resulted in dimer abrogation whilst other substitutions mimicking B*27:09 and B*27:06 at residue 114 had no effect. The manipulation at p116 could result in binding of a different set of peptides to HLA-B27 heavy chain, which consequently could influence

heavy chain assembly rates and/or folding properties of HLA-B27 which determine dimerisation. However, within the B27.SCT, the D116Y change should not alter the peptide repertoire that binds to the heavy chain as the SCT is composed of a heavy chain covalently linked to influenza A virus nucleoprotein (NP) peptide (SRYWAIRTR). The NP peptide naturally contributes to the high affinity interaction with native B*27:05 since aspartic acid at p116 was proposed to form a salt bridge with the positively charged residues R9 located at the C-terminal end of the NP peptide. Moreover, the structural integrity of the B27.SCT constructs was confirmed by their detection with ME1, an antibody that recognises exclusively peptide/ β 2m/heavy chain complexes via immunoblotting and their efficient recognition by CTLs. Intriguingly, studies regarding B27 peptide specificity and crystallography data (Jardetzky et al., 1991) (Madden et al., 1991) suggest that, introduction of tyrosine at p116 most likely disturbs the binding of the influenza NP peptide. Carreno et al. showed that the p116 residue was critical for the interaction of an NP-peptide by HLA-B27 and a D116F point mutation decreased the stability and affinity of the HLA-B27-NP peptide complex (Carreno et al., 1993). This instability was driven by the loss of an acidic charge within the F pocket, leading to a decrease in the allowance for R9 polar residues. Moreover, the presence of Y116 within other B alleles, including the weakly/non-AS associated HLA-B*27:06 subtype, may create an environment that almost exclusively requires non polar amino acids at the C-terminal end of peptides. If so, the D116Y mutation should lead to a diminished affinity between the B27.SCT heavy chain and the NP peptide, but intriguingly at the same time it abrogates dimer formation. Although, it is unlikely that the lower stability of heavy chain- β 2m-peptide complexes per se decrease

the tendency of B*27:05 to misfold and form aberrant heavy chain dimers, it does suggest that the affinity of interaction between antigen and the heavy chain are not the key prerequisite to abrogate HLA-B27 dimerisation. Furthermore, several studies report that non/weakly-AS associated subtypes B*27:06, B*27:09 and B*27:07 form less stable complexes with peptide than HLA-B*27:05 (Galocha and Lopez de Castro, 2010) (Merino et al., 2008) (Williams et al., 2002).

Despite the covalent peptide attachment, the D116Y mutation was able to abrogate dimer formation of the B27.SCT, suggesting that mechanisms other than changing the peptide repertoire could ultimately influence dimer formation. Residues at p114 and p116 also determine whether peptide optimisation is tapasin-dependent (Neisig et al., 1996) (Williams et al., 2002) (Park et al., 2003). In much of the work relating to the role of tapasin and MHC class I have used the HLA-B*44:02 and 05 subtypes that, due to a single D116Y polymorphism, represent subtypes that are tapasin dependent and independent for cell surface expression, respectively (Williams et al., 2002). Recent findings indicate that in addition to editing the peptide repertoire, tapasin-dependent alleles require assistance from tapasin to stabilise their empty peptide binding groove in order to acquire optimal peptide (Zarling et al., 2003) (Garstka et al., 2011). These studies employed the B*44:02 and 05 subtypes and proposed that residue 116 strongly influenced flexibility of the antigen binding cleft (Zarling et al., 2003) (Garstka et al., 2011). Moreover, Fabian et al reported that B*27:05 in comparison with B*27:09, revealed enhanced dynamic flexibility of the α -helices surrounding the peptide binding groove (Fabian et al., 2010). They proposed

that differential dynamic behaviour of the HLA-B27 subtypes at physiological temperature has to be accounted for by an intrinsic attribute of the heavy chain and not for a peptide sequence. If so, a D116Y substitution could alter the flexibility of the HLA-B*27:05 peptide binding groove, which could improve peptide acquisition leading to diminished dimerisation. Thus, the B*27:05 peptide cleft potentially exhibits some degree of disorder, which seems to be improved by D116Y substitution which could further explain why the B27.SCT, despite the linkage of the heavy chain to high affinity peptide remains tapasin dependent, resulting in its slow assembly kinetics and strong association with the PLC. Although, we found that the D116Y substitution does not change the association of HLA-B27 with the PLC significantly, it does not exclude that Y116 is capable of improving stability of the peptide binding groove without altering the interaction between heavy chain and the PLC. HLA-B*44:05, although described as an example of a relatively tapasin independent allele (Zernich et al., 2004), it was shown by Thammavongsa et al. to coprecipitated with TAP (Thammavongsa et al., 2006). Furthermore, the experiments by Springer and colleagues also revealed that both HLA-B*44:05 and -02 bind equally well to the PLC and mutation of B*44:02 at p116 which improved stability of the peptide binding groove, did not change its association with the PLC ((Garstka et al., 2011) and unpublished data).

The presence of HLA-B27 dimers within the PLC and the influence of residues within the F pocket on the ability of HLA-B27 to dimerise indicate that aberrant heavy chain disulphide bond formation may occur within the PLC. Part of the dimerization process would require that HLA-B*27:05 undergoes redox

regulation within the PLC, leading to aberrant interchain disulphide bond interactions. Such redox regulation could be performed by oxidoreductases, such as ERp57 which is found within the PLC (Momburg and Tan, 2002) (Santos et al., 2007). I demonstrated, both native HLA-B27 as well as B27.SCT, form direct and strong conjugates with ERp57. Previously it was demonstrated that ERp57 mainly interacts with the structural cysteine residues within the α 2-domain (Antonίου et al., 2007). Since the main function of ERp57 seems to be disulphide bond reduction it would suggest that the presence of a stable ERp57-HLA-B27 conjugate might reflect engagement of ERp57 in the reduction of the intrachain α 2-disulphide bond which would allow for these cysteines to participate in aberrant interchain disulphide bond formation.

Interestingly, the residue expressed at p116 was found to determine the differential sensitivity of the HLA-B*44:05 and -B*44:02 alleles to ERp57-mediated disulphide reduction within the peptide binding cleft (Kienast et al., 2007). HLA-B*44:02, which relies on tapasin for peptide optimisation and acquisition, was found to be predominantly in an oxidised state within the PLC unless tapasin was absent. In the absence of tapasin, the α 2-domain disulphide of HLA-B*44:02 but not 05 was reduced. The reduction of the α 2-domain disulphide bond was accompanied by a degree of heavy chain misfolding, as detected by its reactivity to HC10, and even formation of high molecular weight heavy chain dimers (Kienast et al., 2007). However, in the presence of tapasin, the reductive properties of ERp57 against the conserved C101-C164 disulphide bond were rendered by recruitment of ERp57 to tapasin (Peaper et al., 2005). My experiments consistently show that within the PLC, ERp57 is able to form stable conjugates with tapasin. This however, does not exclude simultaneous

association of ERp57 with tapasin and heavy chain at the same time, as has been previously detected (Santos et al., 2007). Although, I did not detect such a trimolecular complex what could have been due to limitations of the experimental procedure. Nevertheless, within our group we previously have found that ERp57 is dispensable for HLA-B27 dimer formation since ERp57 depletion does not prevent dimer formation (David Guiliano, submitted). Hence, the interaction between ERp57 and B27 molecules could be indicative of ERp57 being engaged in isomerisation of the disulphide bonds and it may not be the predominant mechanism for HLA-B27 dimer formation. Thus, dimerisation could be mediated by the oxidative action of other oxidoreductase, particularly PDI which has been postulated to be involved in both early and late stage of MHC class I assembly (Kang et al., 2009) (Park et al., 2006).

Furthermore, I show that HLA-B27 molecules that retain the ability to dimerise demonstrate an enhanced association with CNX, which can control both folding and degradation of ER proteins (McCracken and Brodsky, 1996) (Rajagopalan and Brenner, 1994) (Ruddock and Molinari, 2006) (Tector and Salter, 1995) (Molinari et al., 2003) (Vassilakos et al., 1996). These observations would be in line with findings obtained within our group, suggesting that CNX controls HLA-B27 dimerisation by modulating the degradation of these conformers via cellular stress pathways (David Guiliano, submitted). Notably, the stronger association of ERp57 and CNX is not simply the result of different MHC class I protein levels since both HLA-B27 native molecules expressed either under strong and weak promoters remain strongly associated with ERp57 and CNX. Hence,

I show that dimerisation correlates strictly with enhanced chaperone interactions.

I propose that HLA-B*27:05 dimerisation is not solely a spontaneous event resulting from the oxidising environment of the ER. The presence of HLA-B*27:05 dimers within the PLC, the slow folding rate of HLA-B27 despite being physically linked to high affinity peptide and the critical role of residues at p116 in dimer formation, when taken together suggests that B27 aggregation is influenced by peptide acquisition, irrespective of the peptide sequence. Hence, the intrinsic property of HLA-B27 heavy chain driving dimerisation might be accounted for by a disordered state of the peptide binding groove (Figure 3.35). A more open and flexible state of the HLA-B*27:05 peptide binding groove would thus explain the delayed peptide acquisition and slow assembly rate of HLA-B*27:05. Furthermore, such an open flexible state could account for the enhanced exposure and accessibility of the conserved cysteine residues to ERp57-mediated reduction which may occur subsequently to interchain disulphide bond formation which could be promoted by PDI. HLA-B27 dimers stabilised by the PLC could subsequently accumulate leading to ER stress that triggers activation of the UPR, which has been proposed as a mechanism of AS development (Turner et al., 2005) (Turner et al., 2007).

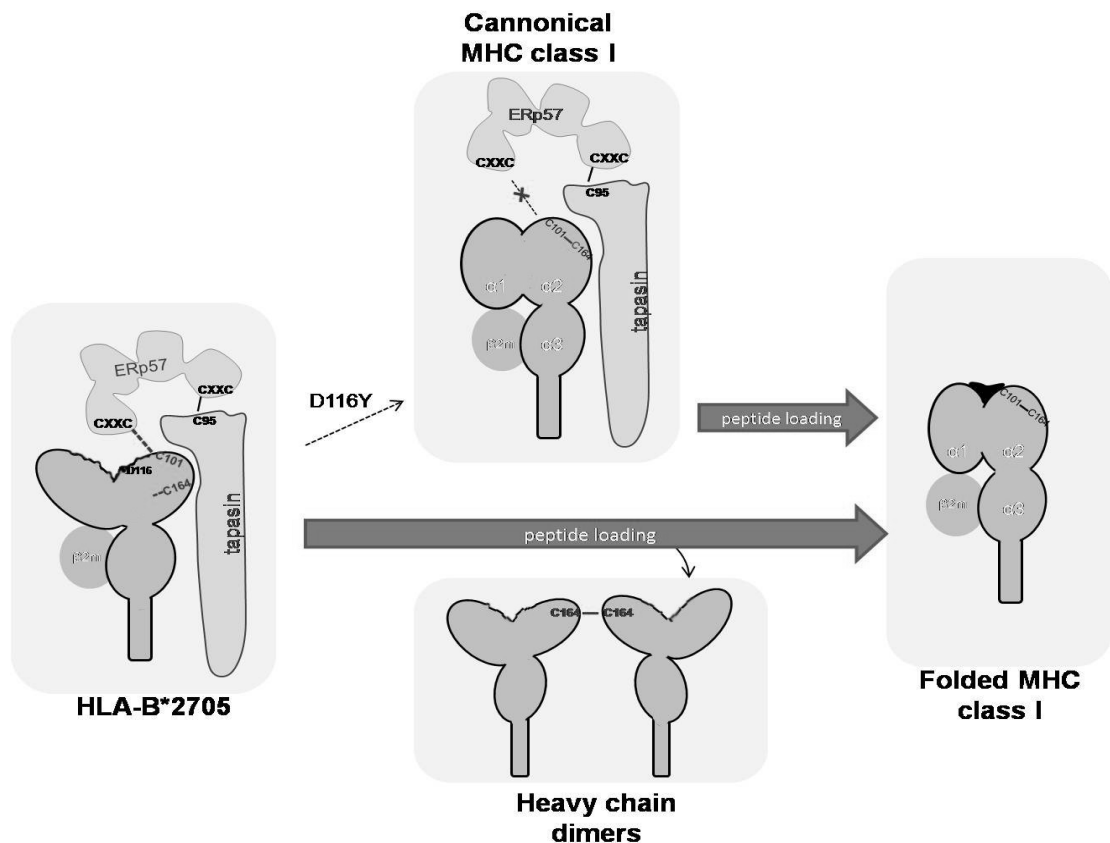


Figure 3.35. Schematic illustrating the plausible mechanism of HLA-B27 homodimer formation within the ER. HLA-B27 most likely possesses a disordered F pocket. Prolonged association of HLA-B27 within the PLC is required in order to stabilise its disordered state by tapasin. A flexible peptide binding groove, together with delayed peptide acquisition could make the $\alpha 2$ -disulphide bond more vulnerable to ERp57 mediated reduction. Reduced C101 and C164 residues could then participate in interchain disulphide bond formation resulting in HLA-B27 dimerisation. The D116Y substitution may stabilise HLA-B27 peptide receptive heavy chain allowing for direct antigen acquisition. Accelerated peptide binding could then influence HLA-B27 conformation, promoting the generation of the fully oxidised heavy chain-peptide complex with a diminished tendency to dimerise.

4 Summary and Conclusions

HLA-B27 is strongly associated with a group of chronic inflammatory arthritic diseases known as the Spondyloarthropaties (Table 1). Based on the enhanced tendency of HLA-B27 to misfold and to form disulphide bonded heavy chain dimers the “misfolding hypothesis” has been proposed. It assumes that accumulation of both misfolded dimeric and monomeric molecules within the ER could trigger disease development by eliciting the UPR (Turner et al., 2007). However, the precise mechanism of dimer formation within the ER remains elusive. Hence, my data provide an insight into the basis for HLA-B27 aggregation.

Implication for HLA-B27 dimer formation

First discovered during in vitro MHC class I folding studies and subsequently reported in transgenic animals, cell lines and AS patient samples, HLA-B27 dimers were originally proposed to form via the unpaired C67 as mutation of C67 prevented dimer formation of recombinant HLA-B27 molecules (Allen et al., 1999). It soon became evident that dimerisation was not solely due to C67-C67 interactions since in the absence C67, dimerisation of HLA-B27 was still evident (Antoniou et al., 2004; Tran et al., 2004) whereas transgenic rats expressing B27.C67S mutant revealed the presence of dimers and develop features of inflammatory arthritis (Taurog et al., 1999) (Tran et al., 2006). My studies provided further evidence against C67 having a major role in B27 dimerisation (Figure 3.5B) and confirmed previous observations by our group (Antoniou et al., 2004), that the conserved structural C164 has a predominant role in dimer

formation (Figure 3.6). I extended previous observations and proposed a novel role for both the conserved structural C101 and C164 in HLA-B27 dimerisation. I demonstrated that C101 and C164 are involved in more complex than direct cysteine-cysteine interactions. They can form distinct dimer structures that differ in the redox state and conformation (Figure 3.12 and Figure 3.19).

My data at least partially addresses the question regarding the nature of the precursors of dimerisation. Although, I cannot exclude that dimerisation involves the newly synthesised heavy chain, my results indicate that HLA-B27 dimers can form at the later stage of the maturation process, particularly within the PLC (Figure 3.26B). Considered together, that HLA-B27 dimers can be detected soon after heavy chain synthesis (Figure 3.26C) but also within the PLC (Figure 3.26B) and that these dimers may engage C164, C101 and C67 (Figure 3.6A-C), most likely in different combinations, a plausible scenario for dimer formation can be proposed: Within newly synthesised heavy chains, C67 and C101 are the first cysteine residues expressed within the ER lumen. Searching for the most thermodynamically favourable state within the oxidising environment of the ER, intensive co-translational folding events could place C67 and C101 in close proximity, thus allowing for intrachain disulphide bond formation. This would leave C164 exposed and available. Further folding events accompanied by the isomerisation activity of oxidoreductases, particularly ERp57, since it was found to form direct and strong interaction with HLA-B27, would then allow for the adoption of canonical MHC class I conformations in which C101 and C164 are buried at the bottom of the peptide binding groove. Proceeding to the PLC the α 2-domain disulphide was proposed to be in a

partially reduced state prior to peptide binding (Antoniou et al., 2007). When oxidised, the vulnerable C67 could form C67-C67 interchain disulphide bonds. However, the slow folding and flexibility within the peptide binding groove (Antoniou et al., 2004) (Fabian et al., 2008) (Fabian et al., 2010) (Hulsmeyer et al., 2004) maybe indicative of the delayed optimal peptide acquisition and suggests that the α 2-domain disulphide remains in a reduced state. This in turn would favour intrachain disulphide bond formation between these two conserved cysteines. Considered together my results indicate that HLA-B27 dimerisation is a complex process and it engages both conserved and unpaired cysteine residues but more importantly it is probably driven by specific peptide acquisition events occurring within the PLC.

My data hence indicate that HLA-B27 dimerisation may involve both newly synthesised heavy chain but also and more predominantly protein that has misfolded during the maturation process. It may involve multiple cysteines and adopt different conformations exhibiting varying degrees of folding/unfolding suggesting that the determination of the crystal structure of HLA-B27 dimers maybe a challenge.

Although my data suggest that C101 and C164 participate in dimer formation, there is also a role for the unpaired cysteines at both p67 and p325 (Figure 3.5B). These cysteines are expressed within the luminal and cytoplasmic tail of HLA-B27 heavy chain hence, in environments that both favour and discourage disulphide bond formation respectively. This suggests that dimerisation may depend on the cellular and environmental context in which cysteines are exposed. Hence, the physiological conditions that give rise and enhance

dimerisation could be related to the alterations in intracellular redox homeostasis. As such, the observation made by Santos et al. that enhanced HLA-B27 dimerisation can be detected in activated dendritic cells (Santos et al., 2008) could result from alterations in the oxidising environment that may occur during DC maturation/activation. In vivo this could take place during activation of DCs in response to inflammatory stimuli (Hacquard-Bouder et al., 2007). If such a condition favours dimer formation, it is likely that other HLA alleles would also gain a propensity to dimerise. Indeed the Santos study detected some dimeric structures in activated B27-negative cells but at a much lower level than B27 dimers (Santos et al., 2008). Furthermore, dimeric structures were found not only in whole cell lysates generated from DC from the peripheral blood of HLA-B27-positive AS patients, but also were identified in B27-negative healthy controls (Campbell et al., 2011). Hence although under certain conditions such as cellular activation and MHC upregulation, other HLA molecules could dimerise as has been previously documented (Capps et al., 1993) (Cresswell and Dawson, 1975) (Boyson et al., 2002). The magnitude of this dimerisation may not be sufficient to elicit downstream effects such as UPR or recognition by effector immune cells (Chan et al., 2005a) (Kollnberger et al., 2007) (Kollnberger and Bowness, 2009) (Turner et al., 2005) (Turner et al., 2007).

Implication for HLA-B27 dimer degradation

The difference in the redox state of distinct HLA-B27 conformers (Figure 3.12) would also be of importance from the perspective of degradation of misfolded B27 molecules. Misfolded MHC class I molecules are targeted, retro-translocated from the ER and degraded in the cytosol via a series of events

known as ERAD (Hughes et al., 1997). Using two immunoevasion proteins encoded by human CMV US2/US11 (Wiertz et al., 1996a) it was illustrated that the removal of the protein substrate from the ER occurs in a redox-dependent manner (Tortorella et al., 1998). MHC class I degradation mediated by US2/US11 was found to be inhibited by agents that altered the redox potential and/or free thiol status such as diamide or NEM (Tortorella et al., 1998). As such, some of the detected HLA-B27 conformers in this study may be due to distinct redox states, which could be more resistant to degradation or inefficiently disposed off by ERAD. This inefficient degradation may readily contribute to the enhanced detection of immature/misfolded protein and B27 aggregation within the ER. Given that the detected novel HLA-B27 conformations with exposed cysteine residues appeared not to be shared by other HLA class I alleles to date (Figure 3.19B), it seems that the complexity of dimeric structures could also be exceptional to HLA-B27. Hence, although to some extent the dimerisation of other alleles could also take place their efficient degradation could prevent them from eliciting pathological responses, whereas HLA-B27 dimers due to their complexity in the redox state and different conformations, could be inefficiently disposed off. Subsequently, these aberrant conformations could well be involved in SpA development by triggering cellular stress responses. Therefore, my studies have important implication for the different redox states and conformations that can occur during the folding of HLA-B27 and the formation of associated dimer structures. However, my data cannot rule out whether and to what extent inefficient HLA-B27 dimer disposition is implicated in disease pathogenesis. Hence, it would be interesting to determine the susceptibility of these different populations to ERAD. Given

that the detection of HLA-B27 conformers is somewhat dependent on distinct redox states which could contribute to inflammatory arthritic disease, intervention strategies manipulating the redox status could be used as potential therapeutics.

Role of peptide in HLA-B27 dimer formation

My data also show that non cysteine residues at p116 expressed within the F pocket that binds the peptide anchor motif at p9, has a profound effect on HLA-B*27:05 cysteine accessibility (Figure 3.24) which in turn correlates with an influence on dimer formation (Figure 3.34). Residue 116 is a prominent polymorphism within both AS and non-AS associated HLA-B27 subtypes. The observed difference in the accessibility to reduction and the redox state of the B27.D116Y mutant (Figure 3.24) could at least partially relate to the peptide repertoire or peptide acquisition. The influence of peptide and/or peptide loading is of importance in light of the recently discovered strong genetic association of ERAP1 with inflammatory disease including AS (Burton et al., 2007) (Brown, 2008) and psoriasis - a skin disease commonly associated with AS (Strange et al., 2010). ERAP1 has two major proposed functions i.e. trimming the peptide precursors to the optimal length for loading onto MHC class I as well as shedding of the proinflammatory cytokine receptors such as TNFR1, IL-6Ralpha, and IL-1RII (decoy IL-1 receptor) (Cui et al., 2002) (Cui et al., 2003b) (Cui et al., 2003a). Several SNPs in ERAP1 have been identified including rs30187 and rs27044 encoding a K528R and Q730E which were shown to influence the catalytical properties of ERAP1 (Evnouchidou et al., 2011) (Kochan et al., 2011). The altered enzymatic properties of these ERAP1

variants were observed towards HLA-B27–restricted antigenic peptide precursors (Evnouchidou et al., 2011) Plausibly, the observed altered catalytic properties of ERAP1 variants may involve mechanisms by which HLA-B27 confers susceptibility to AS. Abnormal peptide processing for instance could affect the redox state of HLA-B27, leading to misfolding and activation of the UPR that in turn upregulates proinflammatory cytokines (Turner et al., 2007) (Turner et al., 2005) (DeLay et al., 2009) (Figure 4.1, pathway 1). Nevertheless, how this genetic association relates to HLA-B27 activity and its folding properties remains to be resolved. Alternatively, the alteration in peptide repertoire that may arise could lead to the expression of β 2m-free B27 heavy chain at the cell surface in the form of either monomer or dimers which could lead to abnormal immune interactions with KIRs and LIRs (Allen et al., 2001) (Chan et al., 2005a). (Kollnberger et al., 2007) (Kollnberger et al., 2002) (Allen and Trowsdale, 2004) (Kollnberger et al., 2004) (Figure 4.1, pathway 2). However, this hypothesis also requires further study as the presence of MHC class I β 2m-free heavy chain at the cell surface does not seem to correlate with subtypes and their susceptibility to AS. The non-disease associated subtype HLA-B*27:06 has a greater proportion of HC10-reactive molecules in comparison with other non-AS associated B*27:09 subtype or AS-associated B*27:04 and B*27:05 subtypes (Goodall et al., 2006). Finally, presentation of altered peptides by HLA-B27 to T cells could potentially elicit autoimmune responses (Laesmaa et al., 1993) (Fiorillo et al., 1997) (Figure 4.1, pathway 3). The latter might be of importance especially in light of studies by Hammer et al.

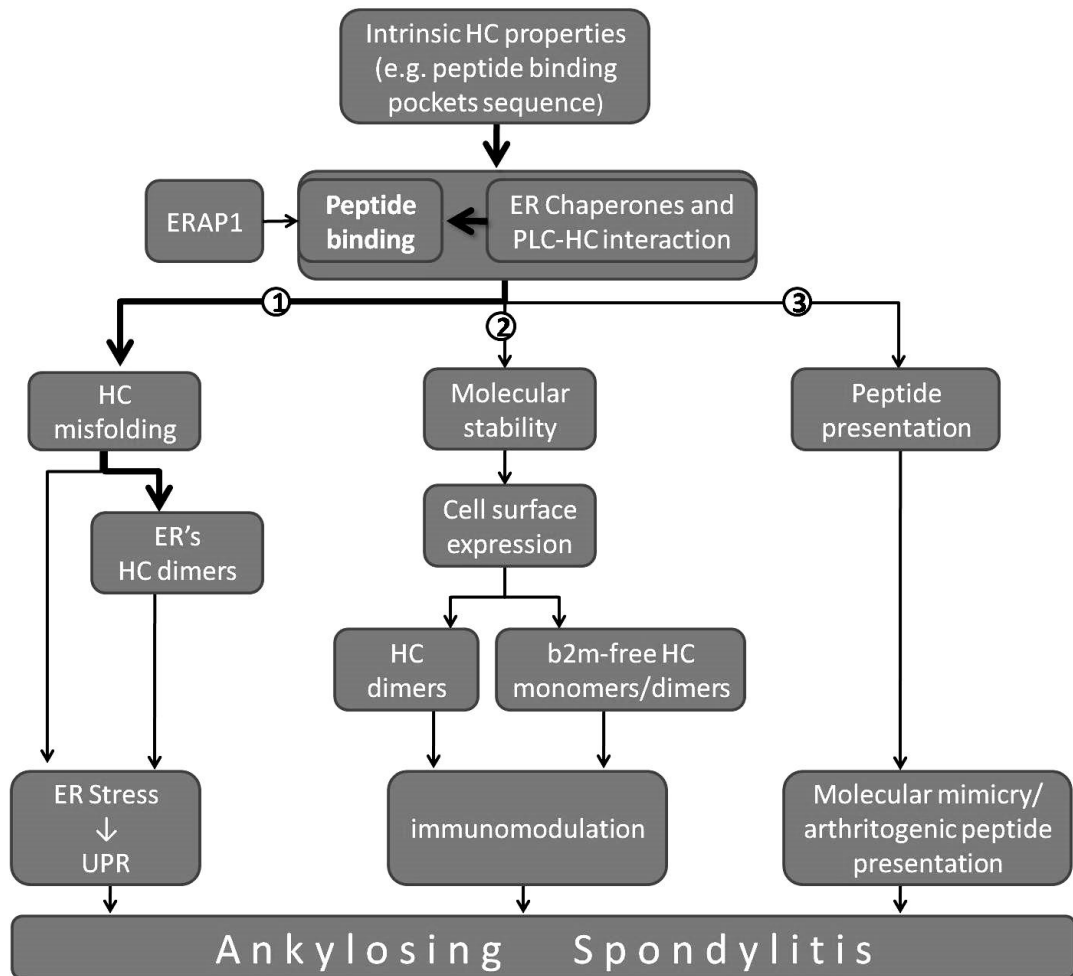


Figure 4.1. Role of HLA-B27 peptide binding properties underlying its association with Ankylosing Spondylitis. The intrinsic properties of the heavy chain, association with ER chaperones/accessory molecules and length of peptide, which depend on proper trimming by the endoplasmic reticulum aminopeptidase (ERAP)1, influence HLA-B27 peptide binding. HLA-B27 antigen acquisition in turn determines other B27 biological features, i.e. (1) folding, (2) stability and (3) ability to present antigens. These three distinct mechanisms can potentially trigger development of Ankylosing Spondylitis. The bold arrows highlight the range of experimental work related to this thesis.

who showed that ERAP1-deficient mouse cells are immunogenic in wild type mice (Hammer et al., 2007).

The consequence of HLA-B27 misfolding

My results indicate that the majority of aberrant HLA-B27 heavy chain forms and conformers, characterised by prolonged cysteine exposure and reactivity, are ER resident (Figure 3.19A). Whether these are a result of aberrant cysteine coupling during heavy chain synthesis and/or inefficient degradation and the subsequent accumulation of aberrant HLA-B27 dimeric forms remains undetermined. Bone marrow derived macrophages from diseased B27-transgenic animals revealed induction of the UPR (Turner et al., 2005). Furthermore, the UPR activation was implied by the detection of BiP overexpression in synovial fluid mononuclear cells from the knee joints of HLA-B27 positive SpAs patients (Gu et al., 2002). Hence activation of the UPR, possibly mediated by HLA-B27 misfolding may play a significant role in AS development. However, misfolding eliciting UPR activation has not been studied rigorously with subtypes other than HLA-B*27:05.

HLA-B27 misfolding eliciting the UPR could be a potential link between recent genetic studies which revealed that the IL23R exhibits a strong association with the SpAs (Burton et al., 2007). IL-23R is expressed by a subset of proinflammatory Th17 CD4⁺ T-cells, whose development is stimulated by cytokines including IL-23. Hence, the IL-23R is involved in the inflammatory response probably by promoting the expansion and survival of Th17 CD4⁺ T-cells that in turn secrete the proinflammatory cytokine IL-17 (Wang et al., 2009)

(McGeachy et al., 2009). Involvement of the IL-23/Th-17 axis in the pathogenesis of AS is further supported by recent work that reported a greater frequency of Th17 cells and an elevated level of IL-17 in the peripheral blood of AS patients in comparison with healthy controls and in synovial fluid (Mei et al., 2011; Melis and Elewaut, 2009) (Singh et al., 2007) (Jandus et al., 2008) (Shen et al., 2009) (Ciccia et al., 2009) (Bowness et al., 2011) (Wang et al., 2009) (Wendling et al., 2007). The studies by Bowness et al. indicate that Th17 cell expansion is promoted by the interaction of cell surface HLA-B27 dimers with KIR3DL2⁺ CD4⁺ T cells (Bowness et al., 2011). However, IL-23 and IL-17 was found to be upregulated also in LPS-stimulated macrophages derived from HLA-B27/human β 2m-expressing transgenic rats that were also undergoing UPR induced by HLA-B27 misfolding (DeLay et al., 2009). The role of the UPR in IL-23 and IL-17 production was also observed in cells treated with pharmacological agents that trigger the UPR (DeLay et al., 2009). However, peripheral blood monocytes, isolated from 10 AS patients in response to LPS, secreted elevated levels of IL-23 without significant UPR induction (Zeng et al., 2011). Because of this contradictory data from animal models of spondylarthritis and AS patients, the relationship between UPR and inflammatory cytokine production require further studies.

Nevertheless, both intracellular and cell surface misfolded HLA-B27 has the potential to trigger activation of the proinflammatory IL-23/IL-17 axis. The link between HLA-B27 and IL-17 production suggests new therapeutic strategies in ankylosing spondylitis/SpA. Particularly, the use of anti-IL-12p40 agents (e.g., ustekinumab) that block both IL-12 and IL-23-mediated effects has been documented to be efficient in psoriasis treatment (Leonardi et al., 2008)

(Mudigonda et al., 2012) (Papp et al., 2008). Hence development of an IL-23 blocking agent could be an effective treatment for AS.

Despite intensive studies the implication of HLA-B27 heavy chain dimers in SpA pathogenesis and whether these structures can be used as potential SpA disease markers remains undetermined. Of importance is whether the misfolding of HLA-B27 monomeric forms or the formation of dimers or both is a pathogenic event and if so, do these events elicit ER stress responses which can lead to the production of proinflammatory cytokines. Particularly, due to the ability of HLA-B27 to misfold and aggregate into dimers, AS can be categorised as a conformational disease together with neurodegenerative disorders or amyloidosis (Herczenik and Gebbink, 2008). Determining the implication of dimeric structures and ER stress in the pathogenesis of the SpAs would be of importance from the perspective of categorising these group of diseases and further treatment.

SCT folding and its implication

Due to physical linkage of heavy chain with antigenic peptide, SCTs were assumed to bypass most of the cellular restrictions on class I antigen processing and presentation. Hence, SCT was proposed to potentially overcome MHC class I down modulation mechanisms such as those used by viruses and some tumors and for this reason SCTs were suggested to have applications as DNA vaccines against virus infection or cancers (Huang et al., 2005) (Huang et al., 2007) (Hung et al., 2007) (Jaramillo et al., 2004) (Li et al., 2010) (Ordaz et al., 2010) (Cheung et al., 2007).

It was previously demonstrated that the pre-assembled nature of SCT allows for its efficient cell surface expression in a TAP deficient cell lines (Kotsiou et al., 2011) however, little information exists regarding the events of SCT folding. Based on HLA-B27 that is well known to misfold I demonstrates that SCTs are potentially chaperone dependent hence maybe limited in their potential use as vaccine vehicles. HLA-B27.SCT associated with CNX, BiP, ERp57 and tapasin (Figure 3.31), suggesting that it requires assistance during both early and late stages of assembly within the ER. My studies reveal that HLA SCT molecules are prone both to misfolding (Figure 3.29C and Figure 3.30) and can associate with the MHC class I machinery (Figure 3.31 and Figure 3.32A). This further suggests that SCT molecules could be susceptible to down modulatory mechanisms employed by viruses to evade immune recognition, which remain to be formally tested thus far.

No virus was found to target chaperones of the early MHC class I folding pathway. However, the association of SCT with CNX and/or ERp57 that are also a part of the degradation machinery could predispose them particularly for downregulation via US2 and US11. US2 and US11 encode early HCMV viral proteins that target MHC class I for rapid degradation in a process mimicking ERAD (Lilley and Ploegh, 2005) (Loureiro et al., 2006) (Powers and Fruh, 2008) (Wiertz et al., 1996a). US11 links MHC class I heavy chains to the Derlin-1-SE1L “dislocation complex” (Ye et al., 2004) that also associates with ER-resident targeting factors including EDEM1 which can handover substrates for degradation. EDEM1 in turn acts in concert with CNX that recruits ERp57 (Elliott et al., 1997; Frickel et al., 2002; Molinari et al., 2003) (Oda et al., 2003) (Oliver

et al., 1999). SCT association with these two latter molecules could indicate that despite their preassembled nature, SCT may be prone to virus-induced degradation.

Several other viral proteins interfere with the function of the PLC predominately affecting the function of TAP or tapasin. The herpes simplex virus (HSV) ICP47 protein, HCMV US6 gene product or EBV BNLF2a protein exploit the function of TAP through different mechanisms (Ahn et al., 1997) (Fruh et al., 1995) (Hengel et al., 1997) (Hill et al., 1995) (Lehner et al., 1997) (Horst et al., 2009). However they all relay on blocking peptide-dependent, ATP-induced conformational changes in TAP. Since these inhibitors predominantly block peptide transport to the ER, it would be expected that they would have minor effects on the SCT due to its preassembled nature. However the components of the PLC need to be structurally coupled to act cooperatively, hence viral-induced alterations in conformational flexibility of TAP which is normally required for peptide translocation, could perturb the function of other PLC components. This could also be the case for viral immunomodulatory proteins that interfere with the function of tapasin such as the HCMV encoded US3 glycoprotein (Lee et al., 2000; Park et al., 2004). Through the direct association with tapasin, US3 inhibits optimisation of the peptide cargo (Lee et al., 2000; Park et al., 2004). In accordance with the finding that not all HLA alleles are dependent on tapasin for optimal peptide binding, not all MHC class I molecules were found to be equally affected by US3 (Park et al., 2004). Nevertheless, tapasin acts in concert with ERp57 and the proper function of tapasin, especially its conformational state, is crucial to inhibit the ERp57 escape pathway which could control the final redox

state of MHC class I heavy chains (Santos et al., 2007). Hence, although the precise mechanism of US3 function is undetermined, any alteration in tapasin function and/or conformation could affect the redox state of the MHC class I heavy chain. Such redox alterations could lead to misfolding, ER retention and/or degradation irrespective of the presence of the peptide and despite the preassembled nature of SCT molecules. Furthermore, US3 was found to enhance degradation of PDI in an undefined manner that correlated with the an increase in the steady state level of the reduced MHC class I heavy chain (Park et al., 2006). Since presence of PDI seems to be essential for MHC class I oxidation, association of SCT with PDI shown in my study suggests that immunoevasion proteins could interfere with SCT assembly through alterations in the heavy chain redox state.

Hence, it would be of relevance to test the influence of a series of pathogen evasion molecules that can inhibit MHC class I molecules at various stages in the assembly, on SCT folding and cell surface expression to fully evaluate the implication of SCTs as vaccine vehicles.

5 Publications

- **Izabela Lenart**, David Guiliano, Garth Burn, Helen Fussell, Simon J. Powis and Antony N. Antoniou, “HLA-B27 forms structurally distinct endoplasmic reticulum resident dimers via cysteine 101 and 164” *Antioxidants and Redox Signalling* 16, 33-43 2011 (publication included full text below).
- Helen Fussell, Darren Nesbeth, **Izabela Lenart**, Elaine C. Campbell, Sarah Lynch, Susana Santos, Keith Gould, Simon J. Powis, and Antony N. Antoniou “Novel Detection of *In Vivo* HLA-B27 Conformations Correlates With Ankylosing Spondylitis Association.” *Arthritis & Rheumatism*, 58, 3419-3424, 2008
- Simon J. Powis, Darren Nesbeth, **Izabela Lenart**, Helen Fussell, Keith Gould, Antony N. Antoniou “Rapid Acidification and Alkylation; Redox Analysis of The MHC Class I Pathway.” *Journal of Immunological Methods*, 340, 81-85, 2009
- Antony N. Antoniou, **Izabela Lenart**, David Guiliano and Simon Powis “Antigen processing and presentation in the MHC class I and II pathway” Book chapter "Vaccinology: Principles and Practice" (in press).
- Eleni Kotsiou, **Izabela Lenart**, Antony N. Antoniou, Julian Dyson, Keith G. Gould “Dimerisation of soluble disulfide trap single chain MHC class I molecules dependent on peptide binding affinity” *Antioxidants and Redox Signalling* 15, pg 635-644, 2011
- Antony N. Antoniou, David Guiliano, **Izabela Lenart**, Garth Burn and Simon Powis “The redox status of HLA-B27 and the influence on biosynthesis” *Antioxidants and Redox Signalling* Vol 15, 669-684. 2011
- **Izabela Lenart**, David Guiliano, Garth Burn, Helen Fussell, Simon J. Powis and Antony N. Antoniou, “HLA-B27 forms structurally distinct endoplasmic reticulum resident dimers via cysteine 101 and 164” *Antioxidants and Redox Signalling* 16, 33-43 2011
- Antony N. Antoniou, **Izabela Lenart**, David B. Guiliano “Pathogenicity of Misfolded and Dimeric HLA-B27 Molecules” *International Journal of Rheumatology* open access article PMID: PMC3087312 2011
- Malgorzata Anna Garstka, Fritzsche Susanne, **Izabela Lenart**, Hein, Zeynep, Gytis Jankevicius, Louise Boyle, Tim Elliott, John Trowsdale, Antony Antoniou, Martin Zacharias, Sebastian Springer “Tapasin dependence of MHC class I molecules correlates with their conformational flexibility” *FASEB Journal* 25, 3989-98, 2011
- Joanna Giles, Jackie Shaw, Christopher Piper, Isabel Wong-Baeza, Kirsty McHugh, Anna Ridley , Demin Li, **Izabela Lenart**, Antony N. Antoniou,

Katilin DiGleria, Kimiko Kuroki, Katsumi Maenaka, Paul Bowness and Simon Kollnberger "HLA-B27 homodimers and free heavy chains are stronger ligands for LILRB2 than classical HLA class 1" J. Immunology 188, 6184-93, 2012

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