The role of beta-2 microglobulin in the endocytosis of MHC class I molecules

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Statutory declaration

I, Sebastián Montealegre, hereby declare that I have written this PhD thesis independently, unless where clearly stated otherwise. I have used only the sources, the data, and the support that I have clearly mentioned. This PhD thesis has not been submitted for conferral of degree elsewhere.

I confirm that no rights of third parties will be infringed by the publication of this thesis.

Bremen, March 23\textsuperscript{th}, 2015

______________________________
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Abbreviations

AP2: adaptor protein 2
APLP2: amyloid precursor-like protein 2
β2m: beta-2 microglobulin
CTL: cytotoxic T lymphocytes
DRiPS: defective ribosomal products
EEA1: early endosomal antigen 1
EGFR: epidermal growth factor receptor
Endo F: Endoglycosidase F
Endo H: Endoglycosidase H
ER: endoplasmic reticulum
ERAP: ER-associated amino peptidases
FHC: free heavy chains
GPI: glycosylphosphatidylinositol
HLA: human leukocyte antigen
IRAP: insulin-regulated amino peptidase
IL-2: interleukin 2
KIR: killer Ig-related receptors
LAMP1: lysosomal-associated membrane protein 1
LDL: low density lipoprotein
MHC: major histocompatibility complex
MβCD: methyl-β-cyclodextrin
NK: natural killers
PLC: peptide loading complex
TAP: transporter associated with antigen processing
TfR: transferrin receptor
UGT1: UDP-glucose:glycoprotein glucosyltransferase I
1. Summary

In the 1980s and 1990s, a few years after the solution of the first crystal structure of a major histocompatibility complex (MHC) class I protein by Don Wiley and co-workers, there was a boom of papers in which every possible aspect of MHC class I molecules was investigated. Since then, MHC class I molecules have become a model system for the investigation of the regulatory mechanisms of the secretory pathway. These studies have helped to find chaperones dedicated exclusively to fold MHC class I molecules, to find novel functions of well-described ER resident proteins, and to understand how the (cellular) immune response is governed to some extent by the intracellular quality control at the level of the secretory pathway, among others.

During that boom, there were some studies on the biochemistry and cell biology of endocytosis that used MHC class I as a model protein. On the biochemistry of MHC class I endocytosis, there are some remarkable studies that will be described later on in this document. With respect to the cell biology, MHC class I became known to the endocytosis field only after the work of Radhakrishna and Donaldson in 1997, as a marker of a new endocytic route. But the biochemical observations have rarely been correlated with the cell biological ones over the last 25 years.

The purpose of this work is therefore to establish a correlation between the biochemistry (i.e., the protein structure) and the cell biology (i.e., the intracellular transport and modification) of MHC class I endocytosis. I have divided this thesis into three main chapters that deal with the endocytosis of MHC class I molecules.

Chapter 2 is a general introduction to MHC class I molecules, to endocytosis, and to what is known about the biochemistry and cell biology of MHC class I endocytosis.

In chapter 3, I present some experimental evidence about the role of beta-2 microglobulin (β2m) in the endocytic destruction of the MHC class I allotype H-2Kb. The most important conclusion of my work is that the dissociation of β2m from the heavy chain of H-2Kb is the key requirement for the endocytic destruction of the complex. My experiments suggest that the dissociation of β2m from the heavy chain occurs mostly inside the cell, that is, in an endosomal compartment. I show here that in a fibroblast cell line, there are very few MHC
class I free heavy chains (FHC) at steady state at the surface. This is somewhat unexpected, given the wealth of literature describing the presence of FHC at the cell surface of different cell lines, but the apparent discrepancy could be resolved. I have also corroborated that an effect known in the MHC class I field as “empty molecules coming out in the cold” is not correctly named. Rather, the statement should be that “reduced endocytosis causes the low-temperature cell surface accumulation of suboptimally loaded MHC class I molecules”. This accumulation is due to the increased affinity of β2m for the heavy chain of Kb at low temperature.

In chapter 4, I present some avenues that can be followed to continue with this investigation.

Chapter 5 reports on a different but related study that I started but did not conclude. Since β2m became central through my investigation on endocytosis, I decided to investigate the structural features of β2m by fluorescence spectroscopy. In this part of the work, I provide experimental evidence, using the small molecule reducing agent TCEP, that β2m is a flexible protein. The work can be a starting point for a more detailed investigation on protein folding in general and especially the potential to correlate in vitro experiments with in silico molecular dynamics simulations.

Statement of Authorship:

The work described in this thesis is my own, unless specified otherwise.
2. Introduction

2.1. MHC class I

2.1.1. General facts

MHC class I molecules are essential components of the adaptive immune response against viruses and cancer cells. Their best-known function is to present antigenic peptides to cytotoxic T lymphocytes (CTL) at the surface of the infected or mutated cell. Following recognition by the CTL, the infected cell is triggered to undergo apoptosis. Additionally, MHC class I molecules interact with killer Ig-related (KIR) receptors on natural killer (NK) cells [1, 2]. In addition to triggering the killing of an infected cell, MHC class I molecules are essential to T cell development, for example in the medullary epithelial cells of the thymus (for positive and negative selection), in the antigen-presenting cells of the lymph nodes (for homeostatic proliferation), and in professional antigen presenting cells such as dendritic cells and B cells (for T cell activation). Novel functions of MHC class I molecules in activities as different as parent-infant recognition or mate choice in mice have been proposed, but not conclusively demonstrated. Such novel functions may or may not be mechanistically related to their function in the immune system [3, 4].

In mammals, three different gene loci for the MHC class I heavy chain exist. In humans, they are located on chromosome 6, and are called the human leukocyte antigen (HLA-A, B, and C) loci. These loci are highly polymorphic: there are more than seven thousand HLA and related alleles reported in the International Immunogenetics Project database [5]. Murine MHC class I molecules, which are highly homologous in sequence and structure to human alleles, are called histocompatibility-2, with the three loci H-2D, H-2K, and H-2L.

MHC class I heavy chains are type 1 transmembrane proteins. An MHC class I-peptide complex is a trimer that consists of the MHC heavy chain, a light chain, and an antigenic peptide (Figure 2.1). The heavy chain has three domains, designated $\alpha_1$, $\alpha_2$, and $\alpha_3$. The light chain is called beta-2 microglobulin ($\beta_2m$; for a detailed description of $\beta_2m$ see section 5.1). Both the $\alpha_3$ domain and $\beta_2m$ are constant domains with an immunoglobulin-like fold, bound to each other by a non-covalent interaction. They support the $\alpha_1$ and $\alpha_2$ domains, which together form a structural 'super'domain that is designed to bind peptides. This peptide binding groove consists of eight beta strands topped by two alpha helices [6,
Some hypervariable amino acids in the peptide binding groove provide for the binding of a great diversity of peptides.

2.1.1.1. **Biosynthesis and folding of MHC class I**

The folding and assembly of a complete MHC class I trimer is a complex process that requires several steps and dedicated chaperones. It starts with the co-translational insertion of the heavy chain into the endoplasmic reticulum (ER), followed by its N-linked glycosylation [8]. After β2m has bound, the dimer of heavy chain and β2m associates with the components of the peptide loading complex (PLC). The function of the PLC is to help the MHC class I dimer load peptides into the binding groove. The PLC consists of the transporter associated with antigen processing (TAP), the lectin chaperone calreticulin, the protein disulfide isomerase ERp57, and the peptide editing chaperone tapasin [9]. The majority of peptides to be presented via MHC class I is produced by the proteasome in the cytosol, most likely from polypeptides that arise from translation products that are incomplete and/or fail to achieve their native structure, the so-called defective ribosomal products (DRiPS) [10]. Peptides between 8 to 16 amino acids in length bind to the TAP transporter [11] and become translocated into the ER. In the lumen of the ER, peptides are further processed by ER-associated amino peptidases (ERAP) [12]. The selection of the peptides that bind to MHC class I molecules is crucial for the further immune response. For that purpose, the chaperone tapasin exchanges low-affinity peptides on MHC class I (which may bind spontaneously during folding or assembly of the heavy chain/β2m dimer) for high-affinity peptides [13-15]. Once a high-affinity peptide has bound to an MHC...
class I molecule, it is transported to the cell surface, where binding to a CTL elicits an immune response.

2.1.1.2. **Peptide binding to MHC class I**

The selection of a high-affinity peptide that can elicit an immune response at the cell surface is crucial. The peptide ligands of MHC class I molecules are usually eight to ten amino acids long and are bound into the MHC class I peptide binding pocket in an extended backbone conformation [16], although even peptides as short as two amino acids can bind transiently to HLA-A2 and H-2K\(^b\) molecules [17, 18]. Usually, two peptide side chains, called the anchor residues, point down into pockets of the binding groove. These requirements define the length of the peptide and the preferred binding motif of an MHC class I allotype, *i.e.*, a peptide must have defined amino acids in the anchor residue positions to be able to bind to the MHC class I binding groove. The amino and carboxyl termini of the peptide are held in networks of hydrogen bonds that are very similar among all known MHC class I structures. These hydrogen bonds are formed between the peptide side chains, the beta sheet, and both lateral helices of the binding groove (including the \(\alpha_{2-1}\) helix) such that the peptide termini stabilize the structure of the binding groove. As a result, the peptide termini make the largest contribution to the binding energy of the peptide [19].

In cells where TAP is either not present or not functional, there are no, or few, high-affinity peptides available to bind to MHC class I molecules. Consequently, in such cells, most MHC class I molecules are devoid of high-affinity peptides. This fact can be used by the researcher to observe suboptimally loaded MHC class I molecules (*i.e.*, loaded with low-affinity peptides or without any peptides).

2.1.1.3. **MHC class I in the secretory pathway**

In general, after peptide binding, the transport of MHC class I molecules through the secretory pathway starts by their uptake into COP II vesicles (Figure 2.2) [20]. In order to prevent suboptimally loaded MHC class I molecules from reaching the cell surface, there is a post-ER quality control mechanism. It is believed to operate as follows: suboptimally loaded and/or partially unfolded MHC class I molecules, including perhaps free heavy chains, are recognized by the enzyme UDP-glucose:glycoprotein glucosyltransferase I
(UGT1), which transfers a new glucose to the end of the glycan tree of unfolded proteins [21].

The modified proteins are then bound by the lectin chaperone calreticulin in the ER, which recognizes only monoglucosylated proteins. Calreticulin and MHC class I molecules travel together to the cis-Golgi, where the KDEL motif of calreticulin is recognized by the KDEL receptor. The recognition of this motif leads to the retrieval of the MHC class I molecules to the ER in COP I-coated vesicles [22]. Upon return to the ER, unfolded molecules are degraded, or peptide is bound with the help of the PLC, and the terminal glucose is removed through the constitutive action of glucosidase II. When MHC class I molecules that have thus escaped the UGT cycle do reach the medial Golgi apparatus, they acquire resistance to endoglycosidases H (EndoH) and F1 (EndoF1). In a late Golgi compartment,
MHC class I molecules finally become modified by sialylation and then proceed to the cell surface [23].

Like the anterograde transport of MHC class I molecules to the cell surface, their endocytic removal from there is controlled by a cellular quality control mechanism. In order to understand how MHC class I molecules are removed from the cell surface in mechanistic detail, it is necessary to have a general understanding of endocytosis. The following section provides this information.

2.2. Endocytosis

In this thesis, endocytosis of a transmembrane cell surface protein is defined as the cellular process that leads to a decrease in its surface levels. It consists of the three steps internalization, recycling to the cell surface from an internal compartment (potentially), and terminal routing to the lysosomes followed by degradation, as explained in the following.

Eukaryotic cells use endocytosis to internalize extracellular fluids, ligands, nutrients, and their own plasma membrane lipids and proteins. Depending on the cell type and on the nature of the endocytosed material, there are several entry pathways into the cell. For the uptake of fluid material, dead cells, or pathogens, pinocytosis and phagocytosis are used. These mechanisms may be non-selective, i.e., all the material may be internalized in a bulk fashion [24]. The best-known mechanism for the selective removal of transmembrane proteins from the cell surface is receptor-mediated endocytosis. In this mechanism, a ligand binds to its receptor on the plasma membrane, causing a conformational change in the receptor; then, this conformational change is recognized on the cytoplasmic side of the membrane, for example through the dimerization of the receptor, which then triggers signaling cascades that eventually lead to the internalization of the complex. Not all cell types perform every type of endocytosis; receptor-independent fluid phase pinocytosis, for example, is mainly found in phagocytes such as macrophages and dendritic cells, which double as professional antigen presenting cells to the immune system in higher vertebrates.

The initial step in the internalization process is always the formation of an endocytic vesicle in which the cargo is internalized. Receptor-mediated endocytosis can be further classified by the type of vesicle in which the cargo is internalized.
2.2.1. **Clathrin-mediated endocytosis**

This is the most widely characterized endocytic pathway [25]. Following ligand binding to specific receptors at the cell surface, the cytosolic domains of the respective receptors are recognized by adaptor proteins such as the adaptor protein 2 (AP2) and other accessory proteins, such as AP180 and epsin, and then assembled into clathrin-coated pits [26].

Dynamin, a large GTPase, is responsible for fission of tubular membranes to generate free endocytic vesicles [27]. The dependence on dynamin to pinch off vesicles is characteristic of every endocytic pathway. Typically, internalized proteins arrive at early endosomes (see section 2.2.5) after five to ten minutes [28], where they are sorted for either recycling to the plasma membrane or degradation. The most commonly investigated proteins internalized by clathrin-mediated endocytosis are the transferrin receptor (TfR) and the low density lipoprotein (LDL) receptor. The typical example of a cytosolic signal that is recognized by AP2 is Tyr-X-X-Φ, where Φ is any bulky hydrophobic amino acid and X represents any amino acid. MHC class I molecules do not possess any known cytosolic signal that would direct them to the clathrin pathway.

2.2.2. **Caveolae-mediated endocytosis**

Like MHC class I molecules, many other proteins do not contain a clathrin recognition sequence in the cytosolic domain. There are alternative pathways to internalize these proteins, such as caveolae–mediated endocytosis. Caveolae are flask-shaped invaginations at the plasma membrane formed by protein-protein interactions between the monomers of the integral membrane protein caveolin [29]. Caveolae are thought to invaginate and collect cargo proteins due to their lipid composition rather than assembly of any cytosolic proteins [30]. Glycosphingolipids and some viruses use this mechanism for internalization [26]. Like clathrin-mediated endocytosis, caveolae-mediated endocytosis depends on dynamin.

2.2.3. **Lipid rafts**

An additional mechanism that depends neither on clathrin nor on caveolae is the internalization via the so-called lipid rafts. Lipid rafts are defined as stable microdomains of lipid bilayers approximately 50 nm in diameter, enriched in cholesterol, sphingomyelin, glycosphingolipids, GPI-anchored proteins, and some transmembrane proteins [31]. In
contrast to caveolae, lipid rafts are flat and do not show the characteristic omega shape of caveolae. They originate by self-association of highly saturated sphingolipids with cholesterol to pack into a highly ordered lipid phase [29]. The vesicular uptake of extracellular or plasma membrane material via lipid rafts depends on small GTPases such as Arf1, Rho A, Rac, and Cdc42 [32]. Rho A and Cdc42 (dynamin-dependent and independent, respectively) depend on lipid rafts for vesicle formation [33]. Flotillin-1 and -2 are raft-associated proteins that play a role in endocytosis, probably aggregating lipid-associated receptors and some lipids to give rise to endocytic vesicles [33].

2.2.4. **Arf6-mediated endocytosis**

The Arf6-mediated pathway has been elucidated mostly through the investigation of individual receptors that do not have clathrin sorting motifs [34]. Several cell surface proteins that are internalized via this pathway such as MHC class I molecules (for details, see section 2.3.2), the alpha chain of the IL-2 receptor, β-integrins, CD55, and CD59 [34]. This pathway might be important to regulate the membrane availability for migratory cells as well as the recycling of small GTPases to the plasma membrane [35]. ARF6-positive tubular endosomal vesicles are different in their phosphoinositide composition [36] from clathrin-dependent cargo-containing endosomes, although the cargo from the former can reach transferrin-positive compartments (*i.e.*, compartments that contain the cargo of the clathrin-dependent pathway) [37]. The Arf6 pathway seems to be independent of dynamin, with a few exceptions [38].

2.2.5. **Endocytic compartments and marker proteins**

Upon internalization, the incoming cargo can be sorted for degradation or recycling to the cell surface. These processes occur in several different endosomes, whose nature depends in their luminal pH, lipid composition, and marker proteins. In the literature, there are several ways to classify such endosomes. Therefore, I describe here the terminology that is used in this document.

The first compartment where incoming cargo arrives after internalization is the **early endosome** or sorting endosome [39, 40]. Like all endosomes, early endosomes are highly dynamic structures that mature to convert into other types of endosomes [41]. Early endosomes have a luminal pH range of 6.3 to 6.8 [42] and are characterized by the early endosomal antigen 1 (EEA1) and the small GTPase Rab5 (see section 2.2.6) [43, 44]. In a
period of approximately 8 to 15 minutes, early endosomes mature and convert into late endosomes [40]. Late endosomes contain cargo that is destined for degradation by acidic hydrolases. Marker proteins of late endosomes are the lysosomal-associated membrane protein 1 (LAMP1), the small GTPase Rab7, and the mannose 6-phosphate receptor [45, 46]. The luminal pH ranges between 4.9 and 6.0 [47]. The final step in the degradation route are the lysosomes. They receive cargo from late endosomes [48] and degrade it with the help of acid hydrolases. As opposed to late endosomes, lysosomes do not contain mannose 6-phosphate receptor [46], and the luminal pH can reach values down to 4.5 [40].

Not all material that is internalized from the cell surface is degraded immediately. The material that is not degraded is recycled to the cell surface via the endocytic recycling compartment or recycling endosomes. These are mainly tubular compartments with a pH of approximately 6.5 [49], and they are characterized by the presence of the small GTPase Rab11 [24]. There are at least two different routes of recycling: the fast route, in which the material is recycled to the cell surface directly from the early endosome with the help of the small GTPase Rab4; and the slow route, in which the material goes through the endocytic recycling compartment in a process mediated by Rab11 and EHD1, among others [28].

2.2.6. Rab GTPases

The formation of endosomes and the transport of cargo through the endocytic pathway are tightly regulated by the Rab proteins. These are GTPases of 21-25 kDa, evolutionarily conserved, and implicated not only in endocytic trafficking, but also in signal transduction and fusion of membrane bound organelles, among others [50].

The molecular mechanism of Rab function is as follows: upon translation, the Rab protein (currently, there are 70 different Rab proteins reported in humans [50]) associates with a Rab escort protein (REP). Then, the Rab protein is geranylgeranylated at its C terminus. In its GDP-bound state (inactive), the Rab protein is inserted into the corresponding membrane with the help of a GDP dissociation inhibitor (GDI) dissociation factor (GDF). Then, a guanine nucleotide exchange factor (GEF) converts the inserted Rab to its active-GTP-bound state, which is now able to interact with effector proteins. Finally, a GTPase accelerating protein (GAP) binds to the Rab protein to hydrolyze GTP to GDP and
to convert the Rab to the inactive state, leading to its release to the cytosol with the help of GDI [51].

Among the Rab proteins, six are potentially important in the endocytosis of MHC class I molecules. Rab4 controls the rapid endocytic recycling of some proteins (such as the transferrin receptor) from the early endosomes to the cell surface [52]. It is present not only in early endosomes, but also in recycling endosomes [53, 54]. Rab5 is the default marker of early endosomes. It was first shown to participate in transport between endosomes [55] and in the regulation of transport from the cell surface towards the early endosomes [53]. The characteristic example of the dynamic nature of the endosomes is the conversion of Rab5-positive endosomes (early) to Rab7-positive ones (late). Thus, Rab7 is implicated in the maturation of late endosomes, directing the cargo along microtubules and helping in their fusion with lysosomes [50, 56]. In the endocytic recycling pathway, Rab11 regulates recycling through the recycling endosomes [57]. Additionally, Rab11 regulates the transport from the recycling endosomes to the trans-Golgi network, which suggests that Rab11 is involved in the interconnection between the endocytic and the secretory pathway. Rab22 has been shown to regulate the recycling of clathrin dependent [58] and clathrin independent cargo [59]. Finally, Rab14 serves as a link between the secretory and the endocytic pathway, since it mediates the trafficking between the early endosomes and the Golgi [50]. Importantly, Rab14 was found to co-localize with insulin-regulated amino peptidase (IRAP) in a human myeloid dendritic cell in an endocytic compartment. The role of Rab14 in the endosomal compartment where IRAP was found might be to prevent its fusion with the lysosomes, thereby reducing the routing of antigens into an acidic lysosomal environment detrimental for cross presentation [60].

2.3. MHC class I endocytosis

2.3.1. Biochemistry of MHC class I endocytosis

A comprehensive introduction into this topic is found in section 3.2.2. Here are further details not mentioned in that section or an extension of some facts mentioned there.

2.3.1.1. Peptide and β2m role in MHC class I endocytosis

The quality of the peptide, i.e., its binding affinity towards the MHC class I heavy chain, determines the half-life of peptide-class I complexes at the cell surface [61]. When bound
to a high-affinity peptide, MHC class I molecules have a long half-life at the cell surface (more than four hours), whereas suboptimally loaded MHC class I molecules are rapidly (in less than one hour) removed from the cell surface and destroyed in acidic compartments [62]. The higher the binding affinity (or, more precisely, the lower the dissociation rate) of the peptide from the class I/β2m/peptide trimer, the longer the half-life of that trimer at the cell surface [63]. The half-life of a class I molecule bound to optimal peptide at the cell surface is also different between different allotypes, but this has not been thoroughly investigated.

After the peptide has dissociated, the resulting β2m-heavy chain dimers are structurally unstable, which results in the dissociation of β2m [64]. In the case of the murine allotype H-2Ld, the dissociation of β2m from the heavy chain of H-2Ld occurs simultaneously with the loss of peptide [65]. Still, both in wild-type cells and in cells that do not have the TAP transporter, some partially unfolded complexes between heavy chains and β2m at the cell surface are always detectable [65]. When external human β2m is added to murine cells, empty dimers of H-2Dd can bind peptides at the cell surface [65, 66]. This suggests that MHC class I heavy chains can bind exogenous β2m and peptides at the cell surface.

MHC class I trimeric complexes can survive low pH values, such as those found in endosomes, for some time. This is important for the recycling of functional molecules to the cell surface. In activated T cells, β2m dissociates from endogenous MHC class I heavy chains at pH 5.6 15-20 minutes after internalization from the plasma membrane [67]. In MelJuSo cells transfected with HLA-A2-GFP, β2m and peptide begin to dissociate at pH 5.0, as detected with W6/32, a conformation-specific antibody that recognizes human MHC class I heavy chains only when they are bound to β2m. In the same study, MHC class I complexes were detected by W6/32 in lysosomal fractions [68].

MHC class I/peptide trimers can return from endocytic compartments to the plasma membrane. This was first demonstrated by Mikael Jondal and co-workers when they treated murine TAP-deficient cells with glycopeptides; after digestion of the cell surface class I molecules with pronase and incubation at 37 °C for some time, they were able to detect H-2Db molecules bound to the glycopeptide at the cell surface, concluding that they must have come from the inside of the cell [69].
The same group postulated that there might be a selective recycling of MHC class I molecules that are not associated with β2m [70]. This was based on two observations. First, they incubated RMA-S cells with human β2m (hβ2m) in serum-free medium; this increased the cell surface expression of H-2D\(^b\). When the experiment was repeated in the presence of chloroquine (a lysosomotropic agent that prevents endosomal acidification), no such increase upon β2m addition was seen. This suggested to the researchers that β2m-receptive free heavy chains had been generated in acidic internal compartments and returned to the cell surface. Second, when they followed a low temperature incubation overnight with hβ2m incubation, they observed that mouse β2m was present at the cell surface, but human β2m was not. They concluded that the recycled heavy chains bind external human β2m.

### 2.3.1.2. The role of the cytosolic tail of MHC class I in its endocytosis

The cytosolic tail of MHC class I molecules does not contain any known motifs for clathrin adaptor proteins. However, there is evidence that the cytosolic tail of MHC class I is involved in its endocytosis.

It was reported early that a set of cytosolic tail mutants of H-2L\(^d\) is not phosphorylated in vivo, compared to full-length H-2L\(^d\). Only after treatment with phorbol myristate acetate (a reagent that activates phospholipid-dependent protein kinase C and AMP-dependent protein kinase A), phosphorylation of the cytosolic tail occurred. In this case, phosphorylation of the cytosolic tail was necessary but not sufficient to trigger H-2L\(^d\) endocytosis [71].

The exons 6 and 7 of MHC class I genes encode the cytosolic tail of the protein. Deletions in the cytosolic tail affect MHC class I endocytosis. HLA-A2 molecules that lack the exon 7 product failed to be internalized in a biochemical assay [72]. In dendritic cells, the deletion of 13 amino acids encoded by exon 7 leads to a significant enhancement of the antiviral CTL response in vivo [73], suggesting that internalization of these molecules is decreased. In addition, short-tailed non-classical MHC class I molecules (HLA-G, HLA-Cw 6/7) that were expressed in .221 cells have a longer life time at the cell surface than classical allotypes (HLA-A2 and HLA-Cw 3/4), as assessed by internalization of the antibody W6/32 [74]. Taken together, the data suggest that the cytosolic tail of class I might increase its rate of endocytosis.
In agreement with these findings, Capps et al. have shown that lack of tyrosine 320, which is present in the cytosolic tail of several MHC class I allotypes, impairs the endocytosis of HLA-B27 molecules [75].

The extracellular domain has also been reported to influence the cytosolic tail. An example of this is that association of β2m with the extracellular domain of HLA-B38 causes a change in its cytosolic tail, preventing the binding of the antiserum ABR2 (raised against a ten amino acid peptide of the cytoplasmic tail of HLA-I class I heavy chains) to the majority of the molecules in a detergent lysate. This has also been observed for murine MHC class I heavy chains. How this effect works in molecular terms remains unclear [76].

In summary, the cytosolic tail of MHC class I molecules plays a role in their endocytosis. It remains to be elucidated exactly which step it is necessary for, and what interaction partners can associate with it.

2.3.1.3. Oligomerization of MHC class I molecules and endocytosis

It is conceivable that there is a binding partner that triggers the internalization of MHC class I complexes at the cell surface. Indeed, a few MHC class I allotypes, such as HLA-B*27:05, can oligomerize at the plasma membrane via disulfide bonds formed between unpaired cysteines present in the peptide binding groove [77, 78] and interact in cis with other membrane receptors [77, 79]. In order to form MHC class I dimers, the dissociation of β2m from the heavy chain is generally required [79]. Such homo- or heteromeric oligomerization of MHC class I molecules at the cell surface has been suggested as a mechanism to trigger their endocytosis [80]. In support of this hypothesis, a polypeptide of 210 kDa was found by co-patching antibodies to MHC class I in Brij-98-resistant membranes. This polypeptide was suggested by the authors as the first candidate marker for an MHC class I-stabilized lipid raft [81]. Lučín and collaborators have also reported that cell surface empty H-2Ld co-precipitates with membrane proteins that survive in large clusters of NP-40 cellular extracts [80].

Taken together, there is only limited evidence of the role of oligomerization of MHC class I molecules at the cell surface as a signal that drives endocytosis. This current evidence is in line with the idea of differential lipid association and the requirement for the dissociation of peptide and β2m from the MHC class I heavy chain for their endocytosis.
2.3.1.4. The role of the membrane environment in MHC class I endocytosis

It has recently been suggested that the micro-membrane environment of MHC class I molecules directly influences its endocytosis. [82]. Supporting this suggestion, in a study performed in the B-lymphoblastoid cell line M12.C3.F6, MHC class I and MHC class II molecules located to different plasma membrane domains, and were internalized via different endocytic carriers. This was assessed by internalization of monoclonal antibodies bound to MHC class I molecules that were followed by microscopy and by membrane extraction with the nonionic detergent Brij-98 [81].

Peptide-receptive and peptide-occupied MHC class I molecules also apparently occupy different membrane microdomains before internalization [80, 83-85]. This was measured in an experiment where the cells were treated for one minute with by Triton X-100 (the so-called “cold triton extraction”) and then stained with conformation-specific antibodies such as 64-3-7, which recognizes a linear epitope on the 310 helix of peptide-free H-2Ld/\(\beta_m^d\) dimers. (This epitope has been transplanted to several other allotypes, such that similar analyses can be done in other MHC class I allotypes [86]). The experiment result was that 64-3-7 positive MHC class I molecules partitioned into a detergent-resistant sub-domain of the plasma membrane, whereas “fully conformed” MHC class I molecules (i.e., those that are 64-3-7 negative and positive for the respective peptide-dependent antibody, such as 30-5-7) were solubilized by Triton X-100. This was true for HLA-B7, HLA-Cw6, H-2Ld, H-2Dd, and H-2Kd [80]. The authors suggest that in addition to the differential localization of the two forms at the plasma membrane, there are also two different internalization pathways for 64-3-7+ and 64-3-7- MHC class I complexes. In these experiments, 64-3-7+ molecules do not recycle to the plasma membrane [83], which suggests that peptide-free MHC class I molecules are targeted for degradation in the endocytic pathway. Using monoclonal antibodies that recognize unassembled heavy chains of human allotypes gave similar results. In conclusion, Mahmutefendic and collaborators claim that association with \(\beta_m^d\) is not sufficient for the endocytic quality control system to consider HC-\(\beta_m^d\) complexes folded proteins and to return them to the cell surface after internalization.

Cholesterol is essential for the structural stability of membranes that contain MHC class I complexes. This was assessed after removal of cholesterol from isolated membranes with the use of the drug methyl-\(\beta\)-cyclodextrin (M\(\beta\)CD) [81]. But there is almost no difference between the rates of internalization of fully conformed H-2Ld and empty H-2Ld after
cholesterol depletion with MβCD, although empty H-2L<sup>d</sup> was internalized faster when sphingomyelin was removed with sphingomyelinase [83].

In summary, there is some evidence that the different forms of MHC class I molecules are associated with different membrane environments and are internalized via different routes. But it remains unclear how this differentiation would be brought about at the plasma membrane.

2.3.1.5. **Free heavy chains**

After dissociation of β<sub>2</sub>m from the MHC class I heavy chain, the latter becomes a free heavy chain (FHC). Many studies have shown that FHC are present at the cell surface (Table 2.1). In most such studies, researchers used primary lymphocytes, activated lymphoblastoid cell lines, or cell lines activated with cytokines. Traditionally, FHC at the cell surface have been detected by two methods: with monoclonal antibodies against FHC, or with the addition of labeled recombinant β<sub>2</sub>m from the exterior of the cell. In both cases, the signal is read by flow cytometry.

Surface FHC have been postulated to arise either from the transport of FHC to the cell surface via the secretory pathway or through the dissociation of β<sub>2</sub>m from heavy chain/β<sub>2</sub>m dimers at the cell surface. This may be different in different cell types. Literature reports also often do not differentiate between unfolded FHC present at the cell surface, and a partially folded FHC able to bind β<sub>2</sub>m.

The physiological role of FHC at the cell surface – if it exists at all – is not well understood. The best example of how they might be physiologically relevant comes from the studies of the interaction between FHC and the insulin receptor [87]. Edidin and co-workers showed that incubation of cells or liposomes bearing HLA FHC at the surface with excess β<sub>2</sub>m decreased the autophosphorylation of the insulin receptor to control levels [88]. This result suggests that there was an actual interaction between the FHC and the insulin receptor, and that this interaction influenced the function of the insulin receptor.

2.3.2. **Cell biology of MHC class I endocytosis**

MHC class I molecules are internalized in a clathrin-independent manner [89]. They are characteristic clathrin-independent membrane cargo proteins [80, 90]. There are some
exceptions, such as when the amyloid precursor-like protein 2 (APLP2) protein associates with H-2K\textsuperscript{d}, partially diverting it to the clathrin pathway [91]; or in some viral infections,

Table 2.1 List of studies where FHC have been found at the cell surface

<table>
<thead>
<tr>
<th>Cell lines/type</th>
<th>Antibody</th>
<th>$\beta_{2m}$ added</th>
<th>Remarks</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Daudi</td>
<td>W6/32</td>
<td>No</td>
<td>FACS; FHC found even with W6/32</td>
<td>[92]</td>
</tr>
<tr>
<td>DAOY, D283, and D556 medulloblastoma cell lines</td>
<td>HC10</td>
<td>Yes</td>
<td>FACS and histopathology</td>
<td>[93]</td>
</tr>
<tr>
<td>Human T cells</td>
<td>HC10</td>
<td>No</td>
<td>3 days of activation</td>
<td>[94]</td>
</tr>
<tr>
<td>HUT-102 cells = human T cell line</td>
<td>LA45</td>
<td>No</td>
<td>Trypsin effect on FHC.</td>
<td>[95]</td>
</tr>
<tr>
<td>JY cells = human B-lymphoblasts</td>
<td></td>
<td>Yes</td>
<td>Only 5% binding</td>
<td>[96]</td>
</tr>
<tr>
<td>RMA, stimulated splenocytes</td>
<td>KU1,KU2,KU4</td>
<td>No</td>
<td>Increase in the population of FHC upon treatment with ConB</td>
<td>[62]</td>
</tr>
<tr>
<td>Several lymphoblastoid cell lines</td>
<td>HC10</td>
<td>No</td>
<td>FRET assay</td>
<td>[97]</td>
</tr>
<tr>
<td>CR-DS= T-cell line; JY cells, and B-CLL cells= leukemia</td>
<td>HC10</td>
<td>No</td>
<td>Metalloprotease</td>
<td>[98]</td>
</tr>
<tr>
<td>Neuroblastoma (NB) cell lines IMR-32 and LA-N-1</td>
<td>L31</td>
<td>No</td>
<td>After retinoic acid or serum starvation</td>
<td>[99]</td>
</tr>
<tr>
<td>RMA-S</td>
<td></td>
<td>Yes</td>
<td>Overnight, kinetic</td>
<td>[100]</td>
</tr>
<tr>
<td>ELON cells = human B-lymphoblastoid cells.</td>
<td>ABR2,HC10</td>
<td>No</td>
<td>Pulse-chase, Endo</td>
<td>[101]</td>
</tr>
<tr>
<td>RMA-S, T2</td>
<td></td>
<td>Yes</td>
<td>Overnight, kinetic;</td>
<td>[61]</td>
</tr>
<tr>
<td>RMA-S, LKD8</td>
<td>D\textsuperscript{d}:34-2-12S $\alpha_1$; 34-5-8S $\alpha_2$</td>
<td>Yes</td>
<td>Overnight, kinetic;</td>
<td>[102]</td>
</tr>
<tr>
<td>EL4 cells = lymphoma; RMA-S; several other cell lines, splenocytes</td>
<td>M1/42;BBM.1</td>
<td>Yes</td>
<td>FACS, long incubations</td>
<td>[66]</td>
</tr>
<tr>
<td>PBMC stimulated</td>
<td>L31</td>
<td>No</td>
<td>After 3 days, FHC present. Nothing at day 0.</td>
<td>[103]</td>
</tr>
<tr>
<td>Activated T and B cell lines</td>
<td>LA45</td>
<td>No</td>
<td>several HLA allotypes</td>
<td>[104]</td>
</tr>
<tr>
<td>PBMC stimulated T cells</td>
<td>LA45</td>
<td>No</td>
<td>Development of the antibody</td>
<td>[105]</td>
</tr>
</tbody>
</table>

where the cytosolic tail of MHC class I molecules is ubiquitinated by viral proteins, inducing clathrin-mediated endocytosis [106, 107].

The endocytic trafficking of MHC class I molecules has been examined with antibodies that detect the fully folded form of MHC class I molecules. After adding such antibodies to live HeLa cells and five minutes of internalization, MHC class I molecules were found in disperse punctate structures that colocalized neither with the LDL receptor nor with
EHD-1, another marker of the recycling endosomes [108, 109]. At the same time, there was colocalization between MHC class I molecules and the IL-2 receptor α subunit Tac [108], another characteristic plasma membrane protein that lacks known retention or surface targeting signals and that is not associated with clathrin [110]. After 20 minutes, MHC class I molecules were found already associated with EEA1+ early endosomes, and there, they met with cargo that was internalized via the clathrin pathway [109]. After ten hours, MHC class I molecules were in lgp120-GFP+ late endosomes [109].

With respect to the recycling of MHC class I molecules, it was shown that endogenous MHC class I molecules (detected with W6/32) colocalize with the small GTPase Arf6 in tubular recycling endosomes in HeLa cells [110]. The hydrolysis of GTP bound to Arf6 is required for the internalization step, whereas GTP-for-GDP exchange allows the recycling of internalized proteins to the plasma membrane [108]. MHC class I molecules destined for recycling have been found in recycling endosomes that contain not only Arf6, but also EHD-1 [108]. Upon overexpression of EHD-1, the recycling of MHC class I molecules from these tubules towards the plasma membrane was enhanced. In the absence of Arf6, there was homotypic fusion of the endosomes that contain MHC class I molecules, hindering their arrival at EEA1+ and Rab5+ early endosomes [109]. When Rab22 was depleted from the cells with siRNA, both the formation of tubular recycling endosomes and the recycling of MHC class I molecules in HeLa cells were impaired, whereas inhibition of Rab11 with a dominant negative mutant impaired the recycling of MHC class I but not the formation of tubular recycling endosomes [59]. In agreement with these observations, it was reported recently that the depletion of the enzyme diacylglycerol kinase A from HeLa cells by siRNA treatment impairs the formation of tubular recycling endosomes as well the recycling of MHC class I molecules to the cell surface [111].

Overall, even though isolated studies of class I endocytosis exist, there is a lack of correlation between the cell biological events in the endocytosis of MHC class I molecules, and the biochemical alterations in the class I molecules that accompany, or cause, these cell biological events.
2.4. **Aim of the study**

The goal of this work was to identify how peptide-bound and peptide-free MHC class I molecules are differentiated in the endocytic pathway. So far, several mechanistic questions in the cell surface quality control of MHC class I molecules are unresolved:

- What is the molecular difference between empty and peptide-occupied MHC class I molecules that causes the endocytosis of empty class I molecules? What are the signals for internalization and for destruction of MHC class I molecules?
- Where does the sorting between empty and peptide-occupied class I molecules take place? Does it occur at the cell surface, in endosomes, or in both compartments?
- Which cellular factor distinguishes between peptide-bound and peptide–free class I molecules?

The answers to these questions are important, since they will provide fundamental knowledge about the regulation of receptor-ligand complexes at the cell surface and allow us to understand how MHC class I molecules that lack peptides are removed from the cell surface, such that no undesired interactions with CTL are produced. They may also allow researchers to develop MHC class I-based biotechnological applications, as dendritic cell-vaccines, which rely on the presence of MHC class I molecules at the cell surface for a long time.
2.5. References


3. Conformational quality control of MHC class I molecules at the cell surface

3.1. Introduction

This chapter presents experimental evidence about some molecular mechanisms that regulate the endocytic destruction of MHC class I molecules. I initially show the establishment of a model system to study the endocytosis of the murine MHC class I allotype H-2K\textsuperscript{b}. This model system allowed me to identify a critical role of \(\beta_2\)m in preventing the endocytic destruction of H-2K\textsuperscript{b}. This part of the work is summarized in a submitted manuscript which is reproduced in section 3.2.

This manuscript was written by Sebastian Springer, myself, and Zeynep Hein. The microscopy experiments were performed by Vaishnavi Venugopalan under my supervision. The pulse chase experiment in (Figure 3.1B) was performed by Susanne Fritzsche. Corinna Kulicke performed some of the repeats of the BFA decays, in particular (Figure 3.2A), under my supervision. Apart from the generation of the cell lines (Zeynep Hein) and the Y3 microscopy experiment (Vaishnavi Venugopalan), I performed every experiment at least once myself.

In the final part of this chapter (section 3.3.), I show some experiments in which the endocytosis of different proteins was evaluated, as well as individual experiments in which the roles of Rab5 and Rab11 in the endocytosis of H-2K\textsuperscript{b} were investigated. Most of the experiments in that section were done only once and therefore should be repeated in the future. The Rab11 experiments were performed by Mohammed Al-Balushi under my supervision, and the Rab5 experiment was performed by Vaishnavi Venugopalan under my supervision.
3.2. Dissociation of beta-2 microglobulin determines the surface quality control of the MHC class I molecule H-2K\textsuperscript{b}

Sebastián Montealegre\textsuperscript{1}, Vaishnavi Venugopalan\textsuperscript{1}, Susanne Fritzsche\textsuperscript{1}, Corinna Kulicke\textsuperscript{1}, Zeynep Hein\textsuperscript{1, 2}, and Sebastian Springer\textsuperscript{1, 2}

*Running title: K\textsuperscript{b} endocytosis is linked to β\textsubscript{2}m

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3.2.1. Abstract

Major histocompatibility complex (MHC) class I proteins, which in all nucleated cells present antigenic peptides at the surface to cytotoxic T lymphocytes, are endocytosed and destroyed rapidly once their peptide ligand has dissociated. The molecular mechanism of this cellular quality control process, which prevents re-binding of extraneous peptides and thus erroneous immune responses, is unknown. To identify the biochemical steps involved in this process, as well as their cellular location, we have followed the removal of optimally and suboptimally peptide-loaded murine H-2K\textsuperscript{b} class I proteins from the cell surface. We find that the binding of their light chain, beta-2 microglobulin (\(\beta_2\text{m}\)), protects them from endocytic destruction. Thus, the extended survival of suboptimally loaded K\textsuperscript{b} molecules at 25 °C is due to decreased dissociation of \(\beta_2\text{m}\). Since all forms of K\textsuperscript{b} are constantly internalized but little \(\beta_2\text{m}\)-receptive heavy chain is present at the cell surface, it is likely that \(\beta_2\text{m}\) dissociation, and recognition of the heavy chain for lysosomal degradation, takes place in an endocytic compartment.

**Keywords:** major histocompatibility complex class I, endocytosis, beta 2-microglobulin, surface quality control

3.2.2. Introduction

Cytotoxic T lymphocytes (CTL)\(^1\) play a major role in adaptive immune responses against viruses and tumors. The recognition and elimination of infected or transformed cells by CTL is initiated by major histocompatibility complex class I (MHC class I) molecules, which present endogenous self and non-self peptides at the surface of target cells. The immunogenicity of presented peptides correlates strongly with the stability of the class I/peptide complex [1]. Stable binding of peptides to class I molecules is mediated by the chaperone tapasin and ensured by the cellular quality control mechanisms located in the endoplasmic reticulum (ER) [2-4]. Accordingly, complexes of low stability dissociate rapidly, and class I molecules are retained in the cell [5]. Even though under normal conditions, all class I molecules at the cell surface are comprised of the heavy chain, the light chain beta 2-microglobulin (\(\beta_2\text{m}\)), and a tightly bound peptide, some suboptimally loaded class I (i.e., without any peptide or with a low-affinity peptide) do emerge from the cell both in mutant and wild type cells [6-10]. In addition, trimeric complexes dissociate after reaching the cell

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\(^1\) Abbreviations: \(\beta_2\text{m}\), beta 2-microglobulin; BFA, brefeldin A; CTL, cytotoxic T lymphocytes; EndoF\textsubscript{1}, endoglycosidase F\textsubscript{1}; EEA\textsubscript{1}, early endosomal antigen 1; ER, endoplasmic reticulum; HA, hemagglutinin; MHC, major histocompatibility complex; scK\textsuperscript{b}, single-chain dimeric K\textsuperscript{b}; TAP, transporter associated with antigen processing.
surface, and the resulting class I heavy chains without peptide, and/or β2m are rapidly internalized [11] and degraded in acidic compartments [12]. Such class I molecules can be rescued from internalization by addition of a high-affinity peptide, indicating that the dissociation of the peptide from the ternary complex triggers the surface removal process [7, 10]. Following the dissociation of the peptide, suboptimally loaded heterodimers become unstable, and dissociation of β2m occurs [13-16]. At the surface of some cell types, class I heavy chains can form homooligomers but these cannot rebind peptides in the absence of β2m [8, 17, 18]. The rapid removal of suboptimally loaded class I molecules at physiological temperatures [10, 19] suggests a yet undescribed cellular process for their specific recognition. At low temperature (22-26 °C), this mechanism appears not to work for the suboptimally loaded murine allotypes, since they accumulate at the cell surface [11]. This is not only because suboptimally loaded class I molecules can escape from ER/Golgi quality control but mainly due to their reduced endocytic removal and destruction [20].

Internalization of class I molecules occurs in a clathrin-independent manner [21, 22]. This mechanism seems not to distinguish between different conformations since peptide-bound class I molecules are endocytosed spontaneously and continuously despite their long life time at the plasma membrane [23-26]. It is therefore possible that the recognition process mentioned above takes place inside the cell.

In this study, we have set out to determine which form of the murine class I allotype H-2K^b is recognized for endocytic destruction, and where in the cell this recognition occurs. Our results support the simple model (Figure 3.6.) that recognition occurs mainly inside the cell, in an early endocytic compartment, and that β2m dissociates there from suboptimally loaded dimers to yield free heavy chains that are then forwarded to lysosomes.

3.2.3. Results

3.2.3.1. Reduced endocytosis causes low-temperature cell surface accumulation of suboptimally loaded class I

In TAP-deficient cells, murine class I molecules are more abundant at the cell surface at 25 °C than at 37 °C [11, 20, 27]. For H-2K^b (K^b) in TAP-deficient RMA-S cells, this 25 °C accumulation is clearly visible (Figure 3.1A). To compare the rates of anterograde transport of K^b at 25 °C and 37 °C, we performed pulse-chase analyses and immunoprecipitated with the
β2m-dependent antibody Y3 [28]. The initial rates of EndoF1 resistance acquisition were very similar at both temperatures (Figure 3.1B, C).

At 25 °C, the labeled K\(^b\) cell surface population continued to increase after 60 minutes of chase, but at 37 °C, it leveled off and then decreased, presumably through endocytosis and lysosomal degradation. This suggests that at 25 °C, suboptimally loaded K\(^b\) molecules are transported to the cell surface at a similar rate, but their endocytic destruction is decreased.

We next tested this hypothesis directly in a Brefeldin A (BFA) decay experiment. We incubated RMA-S cells overnight at 25 °C, added BFA, incubated aliquots at either 25 °C or 37 °C, and measured surface K\(^b\) with Y3 staining and flow cytometry. At 37 °C, K\(^b\) disappeared rapidly from the cell surface with a half-life of about 50 minutes, whereas it
showed no significant decrease after four hours at 25 °C (Figure 3.1D). Like Day et al. [20], we conclude that suboptimally loaded K\textsuperscript{b} accumulates at the surface of RMA-S cells at 25 °C because of reduced endocytosis and not because of increased anterograde transport.

3.2.3.2. **At 25 °C, free heavy chains are still destroyed by endocytosis**

We next tested whether this lack of endocytic destruction at 25 °C is a general cellular feature. We thus compared the endocytosis of suboptimally loaded heavy chain/ \(\beta_2\)m dimers with that of free heavy chains of K\textsuperscript{b}. To enable the detection of free heavy chains, we expressed K\textsuperscript{b} with an N-terminal influenza hemagglutinin (HA) tag in human TAP-deficient STF1 cells [29]. Additional human \(\beta_2\)m (h\(\beta_2\)m) was expressed from the same mRNA with the help of a viral 2A ribosomal skipping sequence [30]; (Figure 3.7A,B). We confirmed that in these cells, too, HA-K\textsuperscript{b} accumulated at the surface at 25 °C (Figure 3.7C), and that HA-K\textsuperscript{b} was not significantly endocytosed at 25 °C (Figure 3.2A). To differentiate dimers and free
heavy chains, we forced the dissociation of hβ2m from cell surface Kβ by incubating the cells at pH 2.6 for 5 minutes, such that signals for both Y3 (for Kβ with bound h β2m) and W6/32 (for STF1-endogenous human class I with bound h β2m) dropped to 40%, whereas the signal of the HA antibody (which recognizes all forms of HA-Kβ) remained constant. As a control, the signal for HC10 (which recognizes human free heavy chains) increased to 3.5 times its preincubation level (Figure 3.2B). Thus, our acid incubation generated significant amounts of free heavy chain.

We then followed the Y3, HA, and HC10 signals in a BFA decay experiment. After one hour, approximately 40% of HA-Kβ molecules were endocytosed, as well as 30% of the human free heavy chains. In contrast, the HA-Kβ/β2m dimers that remained after the acid treatment remained at the cell surface entirely (Figure 3.2C). As a control, both Y3-positive and HA-positive populations were rapidly endocytosed at 37 °C (Figure 3.2D).
The removal of free heavy chain from the cell surface shows that endocytic destruction is not generally blocked at 25 °C, in agreement with earlier reports [31]; thus, at this temperature, a selective process – of unknown molecular mechanism – must be operating to retain suboptimally loaded HC/hβ2m dimers at the surface, or to return them to the surface from an early endocytic compartment. Since at 37 °C, suboptimally loaded class I are rapidly degraded (Figure 3.1D), one might assume that at 37 °C, this retention process does not function. Alternatively, at 37 °C, suboptimally loaded class I might rapidly lose their bound β2m and thus become substrates for the endocytic degradation of free heavy chains.

3.2.3.3. At 37 °C, β2m dissociation limits the rate of endocytic destruction of H-2Kb

We next figured that if the latter hypothesis is correct, then suboptimally loaded class I molecules might be resistant to endocytic destruction at 37 °C as long as their β2m was bound. We thus decided to test in BFA decay experiments two variants of Kb with stronger β2m association, expecting that they would last longer on the cell surface, in suboptimally loaded form, than wild type Kb. First, we tested Kb(Y84C/A139C) (=KbY84C [32], which has an especially high affinity to β2m, and second, the single-chain dimeric Kb (scKb), in which hβ2m is covalently attached to the amino terminus of the heavy chain by means of a long glycine/serine linker (Figure 3.7A,B). Both KbY84C and scKb were very resistant to endocytosis at 37 °C, suggesting that β2m association is sufficient to prevent endocytic destruction of class I (Figure 3.3A, B; Figure 3.8).

If β2m association protects Kb from endocytic destruction, then the simplest hypothesis to explain the 25 °C surface accumulation is that at 25 °C, β2m dissociation from the Kb heavy chain is slower than at 25 °C. To test this hypothesis, we metabolically radiolabeled STF1/Kb cells, lysed them with detergent, immunoprecipitated with the antibody BBM.1 (which recognizes both free and class I-bound hβ2m), and incubated the beads at 25 °C or at 37 °C for up to 30 minutes. At different time points, we quantified the remaining bead-bound heavy chains by SDS-PAGE and autoradiography. Strikingly, after 30 minutes of incubation at 37 °C, 75% of HA-Kb had dissociated from β2m, whereas at 25 °C, there was almost no dissociation (Figure 3.3C, D). Thus, dissociation of β2m from the Kb heavy chain is significantly faster at 37 °C than at 25 °C.

Taken together, our data suggest that at 37 °C, suboptimally loaded Kb rapidly lose their β2m and thus become targets for endocytic destruction, whereas at 25 °C, the slow dissociation of
β₂m causes their retention at the cell surface or their retrieval from an early endocytic compartment. Thus, β₂m dissociation is the rate-limiting step for the endocytic destruction of Kᵇ.

### 3.2.3.4. Dissociation of β₂m takes place in an intracellular compartment

We next investigated where in the cell β₂m dissociation occurs. We hypothesized that if it occurred at the cell surface, then the resulting free heavy chains should be detectable because they can bind exogenous β₂m. First, we incubated the STF1/Kᵇ cells overnight with 7 µM or 37 µM recombinant hβ₂m and found that the Kᵇ levels (measured with the β₂m-dependent Y3 antibody by flow cytometry) had increased considerably (Figure 3.9B). This confirms published data that free heavy chains exist at the cell surface that can bind to exogenous β₂m [7, 8, 33-35]. To estimate the amount of β₂m-receptive free heavy chains at steady state, we next incubated STF1/Kᵇ cells grown at 25 °C or at 37 °C with 7 µM or 37 µM hβ₂m (82 and 434 µg/ml, respectively) on ice (Figure 3.4A). To our surprise, we did not find any increase at all in the Y3 signal after hβ₂m incubation; the same was true in RMA-S cells (not shown). We thought that the lack of hβ₂m binding was perhaps due to rapid denaturation or endocytosis of the free heavy chain, and so instead of adding hβ₂m just before antibody staining, we cultured the cells in the presence of BFA and hβ₂m for up to four hours. We expected that the
exogenous $h\beta_2m$ would bind to the freshly generated free heavy chain and extend the lifetime of suboptimally loaded $K^b$. Remarkably, excess $h\beta_2m$ addition slowed down the decrease of $K^b$ only slightly (Figure 3.4B).

Taken together, the data suggest that overall surface levels of $K^b$ can indeed be stabilized by incubation of cells with $h\beta_2m$ over a long time, but that at any given time point, the steady-state level of $\beta_2m$-receptive free heavy chains is very low. Since a given cohort of surface $K^b$ is not efficiently rescued by exogenous $\beta_2m$, we conclude that dissociation of $\beta_2m$ from the heavy chain occurs mainly inside the cell, perhaps in an early endosomal structure.

### 3.2.3.5. Peptide-bound and suboptimally loaded $K^b$ access an early endocytic compartment

We next reasoned that, if dissociation of $\beta_2m$ from the $K^b$ heavy chain occurs inside the cell, then peptide-empty or suboptimally loaded $K^b$ dimers should be able to access these internal compartments, perhaps cycling between the cell surface and an endocytic compartment. Such internalization was shown previously for H-2Ld [36]. To see whether it occurs in our system, we incubated STF1/$K^b$ cells at 25 °C to accumulate dimers at the cell surface, added the $\beta_2m$-dependent Y3 antibody for five minutes, and then washed the cells and shifted to 37 °C. At different time points, we then fixed the cells and stained with secondary antibody. We observed that within minutes of the temperature shift, $K^b$ moved to punctate structures that colocalized with the early endosomal marker EEA1 (Figure 3.5A). The same movement and colocalization was observed when we left the cells at 25 °C after antibody incubation, albeit at longer times. These results suggest that even at 25 °C, when the cell surface levels of class I appear constant over time, $K^b$ dimers are internalized, presumably returning to the cell surface.

To investigate the internalization of peptide-bound $K^b$, we then repeated the experiment but added SIINFEKL peptide to the cells prior to antibody binding. At 37 °C, we found colocalization with EEA1 after 30 minutes (Figure 3.5B). Thus, the simplest model to explain our observations is that suboptimally loaded dimers and peptide-bound trimers both travel from the cell surface to an endocytic compartment and from there either return to the cell surface or – perhaps concomitant with the loss of peptide and $\beta_2m$ – move on towards lysosomal degradation.
Figure 3.5 Peptide bound and suboptimally loaded K\(^b\) access an early endocytic compartment.

STF1/K\(^b\) cells were incubated overnight at 25 °C; next day, A. cells were incubated with Y3 five minutes at 25 °C; the antibody was washed off, and internalization was followed for 10 and 60 minutes at 25 °C (middle panel) or 10 and 30 minutes at 37 °C (lower panel). After each time point, cells were permeabilized and stained with EEA1. B. Cells were pre-incubated with SIINFEKL, then with Y3, and internalization was followed as in A. Scale bar: 20 µm
3.2.4. Discussion

In this paper, we have asked how suboptimally peptide-loaded dimers (called dimers in the following) of H-2K\textsuperscript{b} heavy chain and β\textsubscript{2}m are selected for endocytic destruction from the plasma membrane. Our results are most parsimoniously explained by the model in Figure 3.6: Dimers, along with trimers (K\textsuperscript{b}/β\textsubscript{2}m complexes with high-affinity peptide) and free heavy chains, are internalized from the plasma membrane (arrows ①, ②, ③) and reach an endocytic compartment. There, dimers lose their β\textsubscript{2}m (④), and the resulting free heavy chains travel on towards lysosomal destruction (⑤), whereas trimers with stably bound peptide are returned to the cell surface (⑥). At lower temperatures (such as 25 °C), dissociation of β\textsubscript{2}m is much slower and dimers return to the surface (⑦), resulting in an increase in the surface level of K\textsuperscript{b}.

Internalization [22, 24, 25, 37-40] and subsequent cell surface return [26, 41-44] of β\textsubscript{2}m-bound class I molecules have been shown by several groups. These experiments have used β\textsubscript{2}m-dependent conformation-specific antibodies for class I such as W6/32 (for HLA) and Y3 (for K\textsuperscript{b}), which also bind to suboptimally loaded dimers [45, 46]. In contrast, explicit demonstrations of internalization of peptide-bound class I [47, 48] and its return to the surface [48, 49] are rare.

In agreement with previous data for human class I [26], our microscopy suggests that at steady state, relatively little K\textsuperscript{b}/SIINFEKL trimer is inside the cell (Figure 3.5), but this does not demonstrate differential internalization of dimers vs. trimers from the cell surface; rather, the return of the trimers from endosomes to the cell surface may be very efficient (Figure 3.6, ⑥).

If dimers and trimers indeed spend 5-10% of their time cycling through endocytic compartments, as assumed previously [26], then it is very likely that the dissociation of β\textsubscript{2}m from the heavy chain takes place in acidic endosomes, since it is dramatically accelerated below pH 5.5 [37, 47]. In this scenario, most free heavy chains generated by dissociation of β\textsubscript{2}m might never appear at the cell surface but instead become routed to lysosomal destruction directly from the endocytic compartment where they are generated (Figure 3.6, ⑥). This matches our observation that extended incubation with exogenous β\textsubscript{2}m cannot rescue suboptimally loaded dimers from destruction (Figure 3.4B). Likewise, in the BFA decay experiments with the HA-K\textsuperscript{b} construct in STF1 cells, the HA and Y3 signals decayed at almost the same rate, which suggests that no significant amount of an intermediate HA\textsuperscript{+}Y3\textsuperscript{-}
species of K\textsuperscript{b} (i.e., free heavy chains) is present at either temperature (Figure 3.2D; Figure 3.8E). In our hands, incubation with exogenous β\textsubscript{2}m at 4 °C does not lead to an increase in the surface Y3 signal on STF1/K\textsuperscript{b} cells (Figure 3.4A).

Even though we find very little free heavy chain that is able to bind β\textsubscript{2}m (called in the following β\textsubscript{2}m-receptive free heavy chain) at the plasma membrane at steady state, this does not necessarily contradict older papers that show β\textsubscript{2}m binding to the surface of RMA-S and other (including human) cells [7, 8, 33-35, 50], for the following reasons: First, in the experiments in the literature, cells are mostly incubated with exogenous β\textsubscript{2}m for several hours; in such incubations, bound β\textsubscript{2}m accumulates even if the steady-state amounts of β\textsubscript{2}m–receptive free heavy chain are small (Figure 3.9B). Second, those short-term incubations with β\textsubscript{2}m that are documented in the literature confirm our findings that only small amounts of β\textsubscript{2}m-receptive free heavy chain are present at steady-state [51]. Third, wherever different cell types were compared, the surface amounts of β\textsubscript{2}m–receptive free heavy chains were found to depend on the surface amount of class I (which depends on the cell type, \textit{i.e.}, professional or non-professional antigen presenting cells, and the activation state), and on the growth phase [17, 52]. Fourth, since we used human cells to express K\textsuperscript{b}, the high affinity of h β\textsubscript{2}m to K\textsuperscript{b}
might cause slower dissociation of β2m; therefore, on our STF1/Kb cells, fewer peptide-receptive free heavy chains might be present than in RMA-S [50, 53]. Fifth, in our experiments, we cultured the cells in FCS-containing medium that contains bovine β2m (bβ2m), which binds to murine class I [8, 54]. Thus, any small amounts of free heavy chain that are generated on our cells might rapidly bind bβ2m from the medium and thus not be available to bind exogenous hβ2m. In our experiments, the free heavy chains at the cell surface were not removed by trypsin treatments, since addition of β2m before trypsinization did not change the outcome (Figure 3.9C).

For our conclusion, in summary, it is most important that incubation of STF1/Kb over several hours with additional exogenous hβ2m does not rescue a cohort of suboptimally loaded dimers at the cell surface in a BFA decay experiment (Figure 3.4B), which demonstrates that steady-state levels of β2m-receptive Kb free heavy chains at the surface of STF1 cells are low.

Such low steady-state levels may have two reasons. Either, free heavy chains are indeed generated at the cell surface but, once generated they rapidly denature to lose their ability to bind β2m. Or, alternatively, free heavy chains are mostly generated inside the cell where exogenous hβ2m does not reach them. Since our Kb molecules are tagged with the conformation-independent HA epitope, we were able to show that during a BFA decay, Y3 and HA epitopes decay at almost the same rate, which suggests that in STF1/Kb cells, no significant accumulation of Y3 HA+ free heavy chains exists. Thus, in these cells, dissociation of β2m from the heavy chain most likely occurs mainly in an internal compartment (Figure 3.6, ➃).

The question of how free heavy chains are selected for routing to lysosomes and eventual destruction is still very interesting. Our data suggest a mechanism that acts in the early endocytic compartment (Figure 3.6, ➄). That mechanism may require acidification, since in the presence of the acidification inhibitor concanamycin B, the surface levels of free heavy chain increase [12]. Its elucidation will require substantial further work.

The low temperature surface level increase of murine class I [11] is explained in our model by the significantly slower dissociation of β2m at lower temperatures (Figure 3.2C; Figure 3.3C) and the resulting return of dimers to the surface (Figure 3.6, ➇). This agrees with our previous in vitro experiments, which show that the peptide-empty dimer of the Kb lumenal
domain and β₂m is conformationally stable below 32 °C [55], and with the decrease in the amounts of sialylated Kᵇ free heavy chain that is visible in RMA cells at 26 °C [12].

Finally, it nicely concurs with our model that Lucin and collaborators showed the separation of 64-3-7⁺ (suboptimally loaded) and 30-5-7⁺ (peptide-bound) H-2Lᵈ molecules in endosomes, with the former proceeding to lysosomes and the latter recycling to the surface [48]. Identification of the precise subcompartments in which these processes occur is currently ongoing [56]. It remains to be seen what differences in class I surface quality control exist between cell types, and to what extent cell surface quality control is mechanistically connected to the loading of class I molecules in the endocytic tract that occurs in some cells [56, 57].

3.2.5. Materials and methods

Antibodies, peptides, and reagents. Chemicals were purchased from AppliChem (Darmstadt, Germany) or Carl Roth (Karlsruhe, Germany). Mouse monoclonal hybridoma supernatants Y3 [46], W6/32 [45], HC10 [58], BBM.1 [59], HA 12CA5 [60] have been previously described. Rabbit monoclonal EEA1 was from BD Biosciences (Franklin Lakes, NJ, USA). Anti-mouse conjugated with Alexa-488 was from Dianova (Hamburg, Germany). Anti-rabbit coupled to Cy3 or Cy5 were from Jackson ImmunoResearch Europe Ltd. (Suffolk, UK). The synthetic peptide SIINFEKL was from Genecust (Luxembourg), purified by HPLC, and delivered at >90% purity.

Cells. STF1 cells [29] (kindly provided by Henri de la Salle, Etablissement de Transfusion Sanguine de Strasbourg, Strasbourg, France), STF1/Kᵇ, STF1/KᵇY84C, and STF1/scKᵇ cells were grown at 37 °C and 5% CO₂ in low glucose (1 g/L) DMEM medium (GE Healthcare Europe, Freiburg, Germany) supplemented with 10% FCS (Biochrom, Berlin, Germany), 2 mM glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin. Cells were sub-cultured every 2-3 days. RMA-S cells [61] were grown in RPMI 1640 (GE Healthcare Europe, Freiburg, Germany) supplemented as above. For incubation at 25 °C, CO₂ independent medium (Gibco by Life Technologies, Darmstadt, Germany) supplemented as above was used.

Generation of stable STF1 cell lines. A 2A ribosomal skipping sequence (GSGATNPSLLKQA GDVEENPGP) [30] was inserted between the coding regions of hβ₂m and H-2Kᵇ in pKG5 background [62] using primers containing BamHI
overhangs (Forward: 5’-gat ctg gta cta att tct ctc ttg taa agg cag gag agt ttg agg aaa acc ctt gag ctt tcc ac-3’; Reverse: 5’-gat cgt cga agg tgg tct ttc ctc aac atc tcc tgc ctt tta aag aga gaa att agt agc aca-3’). The hβ2m-2A fusion was sub-cloned upstream HA-H-2Kb in pEGFP-N1 [32] via BamHI and SalI. Both hβ2m and H-2Kb contained their respective signal peptides (SS), since upon cleavage of the 2A sequence the transcript of the H-2Kb molecule is inserted into the endoplasmic reticulum independently of hβ2m. The final construct was cloned into the lentiviral vector puc2CL6IPwo [63] via XhoI and AgeI. STF1 cells were transduced with this construct and selected, as described previously [32, 64-66] generating the stable cell line STF1/Kb. A similar strategy was used to create the cell line STF1/KbY84C. The single chain Kb construct was designed essentially as described [62]. The coding region of the single chain dimer was sub-cloned into pEGFP-N1 via XhoI-AgeI. An HA tag was inserted by site directed mutagenesis (Agilent, Santa Clara CA, USA) after the first GGGGS repeat in the linker region, using primers (Forward: 5’-aca tgg gtg gcg gag gta gtg atc cat atg acg tcc ctt att cag cag gtg gcg gtt ggc ggg ccc-3’; Reverse: 5’-ggg cgg gag cca cgg cca cct gcc taa tca ggg aag tca aag gat aac tac ctc cgc cac cca tgt-3’). Finally, the full gene was sub-cloned into puc2CL6IPwo via XhoI-AgeI, and STF1 cells were transduced as above, to create the cell line STF1/scKb.

**BFA decay experiments.** 1.2x10⁵ cells per well were seeded into 6-well plates 2 days before the experiment, and incubated at 37 °C. The night before the experiment, the medium was changed to CO₂ independent medium, and the cells were incubated in an incubator without CO₂ at 25 °C overnight. Next day, cells were washed in PBS, the medium was changed for supplemented DMEM (for 37 °C experiments) or CO₂ independent medium (for 25 °C experiments) containing 5 µg/mL BFA (and 10 µM SIINFEKL peptide where indicated). Afterwards, cells were incubated at either 25 °C or 37 °C for the indicated time points. To determine the baseline values at t=0 in the presence of peptide, cells were incubated with peptide in CO₂ independent medium at 25 °C for 30 minutes. At t= 4 hours, the cells were trypsinized, harvested, and processed for flow cytometry.

**Acid Wash.** STF1/Kb cells were incubated overnight at 25 °C in CO₂ independent medium as above. Next day, cells were trypsinized, washed, and placed on ice. Cells were resuspended in 500 µL Acidic Buffer pH 2.6 (22 mM Na₂HPO₄ 89 mM citric acid) or in Neutral Buffer pH 7.4 (187 mM Na₂HPO₄ 6.4 mM citric acid) for 5 minutes on ice. Afterwards, the pH was neutralized with 15 mL Blocking Buffer pH 7.4 (93 mM Na₂HPO₄, 0.5% BSA in CO₂ independent medium without FCS); the cells were then washed, resuspended in CO₂
independent buffer without FCS and with BFA, and incubated at 25 °C or 37 °C for the indicated time points. Finally, the cells were washed, and processed for flow cytometry.

_Folding and addition of recombinant β2m._ hβ2m was expressed in E. coli as inclusion bodies, purified, solubilized, and stored at -20 °C, as previously described [55]. 8 mg recombinant hβ2m were refolded into 16 different reactions in refolding buffer (100 mM Tris-Cl pH 8.0, 0.5 M arginine, 2 mM EDTA, 0.5 mM oxidized glutathione, 5 mM reduced glutathione, and protein to a maximum concentration of 250 µg/mL, for a final volume of 2 mL per reaction). After 60 hours, the samples were ultracentrifuged, the supernatant was collected, and the buffer was exchanged for PBS using Vivaspin 2 10 KDa MWCO ultrafiltration columns (VS02H02, Sartorius, Göttingen, Germany). Typical final concentrations yielded 2.3 mg/ml, quantified by the Bradford assay. To verify that the protein was folded, TDTF measurements were performed after every preparation, as described previously [55]; STF1/Kb cells were incubated overnight at 25 °C; next day, a BFA decay at 37 °C was performed, in the presence of freshly prepared hβ2m, at two different concentrations: 7 µM or 36 µM hβ2m. Cells were harvested, stained with Y3, secondary antibodies, and fluorescence was detected by flow cytometry.

_Detection of Free Heavy Chains._ hβ2m was prepared as above. STF1/Kb cells were incubated overnight at 25 °C or at 37 °C. Next day, cells were trypsinized at room temperature, washed, and incubated without hβ2m or with 7 µM or 36 µM hβ2m 15 minutes on ice. Cells were washed, stained with Y3, anti-mouse coupled to Alexa-488, and fluorescence was recorded by flow cytometry.

_Flow cytometry._ Cells were kept on ice throughout the staining process. Cells were fixed with 0.02% NaN₃ in PBS for five minutes, washed, and incubated with primary antibody for 30 minutes. Anti-HA antibody 12CA5 hybridoma supernatant was used in a 1:5 dilution; W6/32, HC10, and Y3 hybridoma supernatants were used undiluted. After primary antibody incubation, cells were washed once with PBS, centrifuged, and stained with anti-mouse coupled to Alexa-488 for 30 minutes. Finally, the cells were washed with PBS as above, and then resuspended in 1 mL PBS for flow cytometry. The living population was determined after staining with fluorescein diacetate (Sigma-Aldrich, St. Louis MI, USA). 3 x 10⁵ living cells were counted on all experiments. The flow cytometer was a CyFlow space (Partec, Görlitz, Germany).
Antibody-mediated internalization. 2 x 10^4 STF1/K^b cells were seeded in cover slips and incubated at 37 °C. After 24 hours, cells were incubated at 25 °C or left at 37 °C accordingly. Next day, cells were washed 3 times with PBS, and incubated with antibodies diluted as above for 5 minutes at room temperature. Then, unbound antibodies were washed, and the cells were incubated for the indicated time points at 25 °C or at 37 °C with the bound antibodies. Cells were then fixed with 4% PFA in 200 mM HEPES, permeabilized with 0.1% saponin, and the secondary antibodies were added. For co-staining, the respective primary antibodies were added after the first secondary antibodies. After washing, the cells were incubated with the second secondary antibodies, and finally the cover slips were mounted.

Confocal fluorescence microscopy. Cover slips were embedded with mowiol onto glass slides. The immunofluorescent stained cells on cover slips were observed under a Zeiss LSM 510 META confocal laser scanning microscope, provided with Argon and Helium-Neon lasers (Carl Zeiss GmbH, Jena, Germany). Images were obtained at a pinhole setting of 1 Airy unit, 63X magnification and a resolution of 1600 × 1600 pixels; they were further analyzed and processed using ZIK-Image J 1.42h (National Institutes of Health, USA).

Pulse-chase experiments. Experiments were done essentially as on [28]. Briefly, RMA-S cells were metabolically pulse labeled for 10 min with [35S]-methionine and cysteine (NEG772002MC Perkin Elmer, Waltham MA, USA) at 37 °C, and aliquots were chased in the presence of an excess of unlabeled Met and Cys for the times and temperatures indicated. Following lysis for 1 hour, and immunoprecipitation with Y3, individual samples were treated with EndoF1, and separated by SDS-PAGE. Proteins were detected by autoradiography, and band intensities were determined by densitometry.

β2m dissociation assay. 1.6 x 10^7 STF1/K^b cells were starved for cysteine and methionine in RPMI without Cys and Met (Sigma-Aldrich, St. Louis, USA), supplemented with 2% FCS, 1% Glu, 1% Pen/Strep for 1.5 hours. Cells were labeled with [35S]-Met and Cys 30 minutes at 37 °C. Afterwards, cells were lysed in Lysis Buffer (50 mM Tris-Cl pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100) one hour at 4 °C. After lysis, the post-nuclear supernatant was immunoprecipitated with BBM.1 antibodies pre-bound to Protein A-agarose beads 30 minutes at 4 °C. The beads were washed three times with Wash Buffer (50 mM Tris-Cl pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.1% Triton X-100), and then distributed into different micro centrifuge tubes. After centrifugation, the supernatant was removed completely, the beads were resuspended in 50 µl Wash Buffer, and the tubes were transferred to heating
blocks pre-warmed at 25 °C or 37 °C accordingly. Finally, the beads were centrifuged, the supernatant was discarded completely, the beads were boiled at 95 °C in Laemmli Sample Buffer, and the immunoisolates were separated by SDS-PAGE. Proteins were detected by autoradiography, and band intensities were determined by densitometry.

**Immunoprecipitation and Immunoblotting.** STF1/Kb, STF1/KbY84C, and STF1/scKb cells were lysed as above, and immunoprecipitated with HA antibodies pre-bound to Protein A-agarose beads. After immunoprecipitation, the samples were treated (or not) with Endo F1, and then immunoblotted against HA. As a control, untransduced STF1 cells were immunoprecipitated under the same conditions. The immunoisolates were resolved by non-reducing SDS-PAGE.

**Data analysis and processing.** Flow cytometry data was obtained with FlowMax and analyzed in FlowJo (Tree Star, Inc., Ashland, US). All calculations were made with Microsoft Office Excel. Graphs were created with GraphPad (GraphPad Software, Inc., La Jolla, USA). Figures were edited with Inkscape (http://www.inkscape.org/). Gels were processed with Image J (National Institutes of Health, USA).

### 3.2.6. Acknowledgments

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### 3.2.7. References


3.2.8. Supplementary Information
Figure 3.7 Generation of novel cell lines to study H-2K^b endocytosis

A. Three different H-2K^b constructs were cloned into a lentiviral vector. Top: HA-H-2K^b WT: signal sequence (SS); hemagglutinin tag (HA); H-2K^b WT heavy chain; 2A ribosomal skipping sequence (GSGATNPSLLKQAGDVEENPGP) (cleavage takes place between N19 and P20, triangle); signal sequence (SS); human β2m. Center: HA-H-2K^b-Y84C: as above, but with an artificial disulfide bond formed between H-2K^b mutated residues Y84C and A139C. Bottom: single chain K^b WT. This construct does not have a 2A sequence, but a GGGGS linker that binds covalently the C terminus of hβ2m and the N terminus of H-2K^b. The HA tag is after the first GGGGS repeat.

B. The cleavage of the 2A sequence is efficient. STF1/K^b, STF1/K^b Y84C, and STF1/scK^b cells were lysed and proteins were immunoprecipitated with an antibody against HA. After immunoprecipitation, the samples were treated (or not) with Endo F1, and then immunoblotted against HA. As a control, untransduced STF1 cells were subjected to immunoprecipitation under the same conditions. C. Surface accumulation at 25 °C also occurs in STF1/K^b cells. RMA-S cells and STF1/K^b WT cells were incubated overnight at 25 °C; next day, the cells were stained with Y3 and anti-mouse secondary conjugated with Alexa 488, and florescence was recorded by flow cytometry.
Figure 3.8 BFA decay experiments in STF1/K^b, STF1/K^b Y84C, and STF1/scK^b cells, detecting with HA antibody.
A and B are equivalent to Figures 3.3A and 3.3B. C BFA decays in STF1/K^b Y84C cells, in the absence of peptide, comparing the Y3 and the HA epitopes. D. As in F for STF1/ scK^b cells. E. as in C for STF1/K^bWT cells. Error bars represent SEM of n = 4 independent experiments.
Figure 3.9 The recombinant hβ2m added to the cells is correctly folded

A. After ultrafiltration, 7 µM hβ2m was measured by TDTF. This is a representative curve of all the experiments where recombinant hβ2m was used. B. STF1/K\(^b\) cells were incubated overnight in the presence of 7 µM or 37 µM recombinant hβ2m. Next day, the cells were harvested, stained with Y3 and anti-mouse secondary antibody conjugated with Alexa 488, and fluorescence was recorded by flow cytometry. C. Trypsinization does not remove preexisting K\(^b\) free heavy chains. As in Figure 3.4A, with the exception of incubation with hβ2m preceding trypsinization. Error bars represent SEM of n=3 independent experiments.
3.3. Additional Data

3.3.1. Covalent attachment of human $\beta_2$m to $K^b$-GFP molecules delays its endocytosis if the HA tag is placed at the C terminus of $\beta_2$m, but not if it is placed at the N terminus

Figure 3.3A shows that when $\beta_2$m is covalently attached to the heavy chain of $K^b$ (single chain construct), its lifetime at the cell surface increases substantially compared to the wild type.

Before the establishment of the single chain $K^b$ stable cell line (STF1/sc$K^b$), I created two HA variants of the single chain construct, namely N´HAsc$K^b$-GFP and C´HAsc$K^b$-GFP (Figure 3.10A). The difference between these constructs is the following: in N´HAsc$K^b$-GFP, the HA tag is located at the N terminus of $\beta_2$m, whereas in C´HAsc$K^b$-GFP, the HA tag is located inside the linker, that is, at the C terminus of $\beta_2$m. Both constructs have GFP fused to the C terminus of $K^b$.

To test these constructs, I transiently transfected STF1 cells and then performed a BFA decay assay at 37 °C in the presence or absence of the high-affinity peptide SL8, and then I stained with Y3. The way to read the composite plot (Figure 3.10B) is as follows: for each individual panel, the y axis shows the amount of $K^b$ at the cell surface and the x axis shows the GFP production, which is equivalent to the total amount of folded $K^b$-GFP produced by the cell. The columns (labeled at the top) are the individual experimental combinations, and the rows (labeled on the right hand side) are the time points that range from 0 to 240 minutes. At time point zero, in the absence of peptides, the cell surface expression of all mutants increases in proportion to the total amount of protein. One can then read each individual experimental combination over time (i.e., down the column); if the slope of the x-y correlation decreases over time, it means that class I molecules became endocytosed (less surface expression); if, in contrast, it remains the same, then endocytosis was slow or not occurring at all.

The data show that in the absence of peptides, sc$K^b$-GFP molecules are remarkably stable at the cell surface (as observed in Figure 3.3A). When an HA tag is placed on the N terminus of $\beta_2$m, N´sc$K^b$-GFP molecules are rapidly endocytosed. Conversely, when the HA tag is placed on the linker, C´HAsc$K^b$-GFP molecules become stable at the cell surface. In all cases, the binding of high-affinity peptides increases the resistance of $K^b$ molecules to endocytosis, as observed in the cells that express low amounts of GFP (Figure 3.10B).
These data indicate that although the covalent attachment of β2m to H-2Kb significantly reduces the endocytosis rate of H-2Kb, it is not sufficient per se to prevent the endocytosis of H-2Kb. A hypothesis that may explain this result is as follows: the presence of the HA tag at the N terminus of β2m might interfere with the proper way of binding of the covalently attached β2m to the heavy chain in an endocytic (acidic) compartment. This would explain why the N’HAscKb-GFP molecules are able to travel through the secretory pathway and reach the cell surface; upon internalization, they reach an endosome, and then – after dissociation of
the covalently attached HA-β2m domain – they are recognized by the (unknown) quality control machinery as a FHC.

3.3.2. **Free heavy chains are not detectable at the cell surface at 25 °C by addition of external recombinant hβ2m**

As observed in Figure 3.4B and Figure 3.9A, hβ2m can be used to detect FHC. To do so, one can add recombinant hβ2m to the cells, stain with an MHC class I conformation-specific antibody, and ask whether there is an increase in the signal compared to the cells to which no hβ2m was added. If there is an increase in the signal measured with the conformation-specific antibody, this will reflect the presence of β2m-receptive (i.e., natively folded) FHC. I thus performed the following experiment: I transfected STF1 cells with a construct that encodes the fusion protein K<sup>b</sup>-GFP, and then I incubated the cells overnight at 25 °C. On the next day, I added different amounts of recombinant hβ2m to the culture medium for one hour at 25 °C. Then, I split the cells into three different sets of samples. In the first sample, I washed the cells and processed them immediately for flow cytometry. At 25 °C, regardless of the amount of recombinant β2m added there was no difference in the fluorescent signal between the samples with or without exogenous hβ2m (Figure 3.11B). In the second set of samples, I incubated the cells with BFA for two hours at 37 °C and then processed them for flow cytometry. Here, K<sup>b</sup>-GFP molecules were endocytosed rapidly regardless of the initial amount of β2m added at 25 °C (Figure 3.11C). In the third set of samples, after washing the cells with medium, I re-incubated them with BFA and recombinant hβ2m for two hours at 37 °C and then processed them for flow cytometry. Here, the endocytosis of K<sup>b</sup>-GFP molecules was reduced in a β2m concentration-dependent fashion (Figure 3.11D) (as observed in Figure 3.4B).

The simplest explanation for these data is that at 37 °C, FHCs are produced very fast (most probably in an endocytic compartment, as discussed in section 3.2.4, such that even relatively large amounts of exogenous β2m cannot prevent efficiently the endocytosis of K<sup>b</sup>-GFP molecules. At 25 °C, either the K<sup>b</sup>-GFP FHCs present at the cell surface are unfolded, and thus, they are not able to bind exogenous β2m, or simply there are no K<sup>b</sup>-GFP FHCs at the cell surface. Thus, the experiment provides further support to the hypothesis that there are very few, if any, β2m-receptive K<sup>b</sup> FHC at the cell surface.
Free heavy chains are not detected at the cell surface at 25 °C by the addition of external recombinant hβ2m

STF1 cells were transiently transfected with a construct coding for the fusion protein Kb-GFP. Four hours after transfection, cells were incubated overnight at 25 °C. Next day, recombinant hβ2m was added to the culture medium for one hour at 25 °C. Then, cells were processed for flow cytometry (A, top panel and B); or, cells were incubated in the presence of BFA for two hours at 37 °C, and then processed for flow cytometry (A, medium panel and C); or, cells were incubated in the presence of BFA and recombinant hβ2m for two hours at 37 °C, and then processed for flow cytometry (A, lower Panel and D). Staining was done with Y3. The figure is representative of three or more independent experiments.
3.3.3. **Endogenous H-2K\textsuperscript{b} and H-2D\textsuperscript{b} free heavy chains cannot be detected at the cell surface of RMA-S cells by the addition of recombinant hβ\textsubscript{2}m**

In an experiment analogous to that shown in Figure 3.4 and Figure 3.9C, I wanted to observe whether in RMA-S cells, I would be able to detect FHC at the cell surface.

To answer this question, I incubated RMA-S cells overnight at 37 °C or at 25 °C, respectively. On the next day, I incubated the cells with 7 µM hβ\textsubscript{2}m (100 µg/ml) for 15 minutes on ice or left them untreated and then processed the cells for flow cytometry. I stained with Y3 or with B22.243 (to detect H-2D\textsuperscript{b}/β\textsubscript{2}m).

At 37 °C, there was very little H-2K\textsuperscript{b} and H-2D\textsuperscript{b} at the cell surface, as expected (Figure 3.12). At 25 °C, there was a substantial increase in the amount of H-2K\textsuperscript{b} and H-2D\textsuperscript{b} at the cell surface. In the presence of 7 µM recombinant hβ\textsubscript{2}m, there was no increase in the fluorescence intensity compared to the untreated samples. For H-2K\textsuperscript{b}, there was no increase in the fluorescence intensity either.

I conclude that in RMA-S, under the examined conditions, there are no FHC that can bind exogenous hβ\textsubscript{2}m. (Note that this experiment was only performed once!) Thus, this experiment supports the data obtained in the STF1/K\textsuperscript{b} cells about the fate of FHC.

3.3.4. **Characterization of STF1/K\textsuperscript{b} cells and STF1/scK\textsuperscript{b} cells**

Before optimizing the dissociation assay (Figure 3.3C, D), it was important to determine the specificity of the antibodies. Thus, I metabolically radiolabelled the newly synthesized proteins in STF1/K\textsuperscript{b} and STF1/scK\textsuperscript{b} cells with \([35]S\) for 10 minutes. After cell lysis, I performed an immunoprecipitation experiment with antibodies against the HA tag of HA-K\textsuperscript{b} WT with W6/32 (endogenous HLA class I molecules) or with BBM.1 (against human β\textsubscript{2}m, both free and bound to MHC class I).

HA-K\textsuperscript{b} WT molecules migrate in the gel more slowly than the endogenous HLA molecules, since the HA tag adds to their molecular weight (compare Figure 3.13, lanes 1 and 2). Both endogenous HLA allotypes and the transduced HA-K\textsuperscript{b} WT were precipitated with BBM.1 (lane 3). The β\textsubscript{2}m band was weak and diffuse (lanes 3 and 5; in further experiments, there were indeed two bands, corresponding to 2A-β\textsubscript{2}m and the endogenous β\textsubscript{2}m, respectively; see Figure 3.3C). The single-chain K\textsuperscript{b} molecules migrated more slowly than the endogenous HLA...
allotypes, as expected, since the heavy chain is covalently attached to β2m (lanes 4 and 6). Both endogenous and single-chain H-2K\(^b\) molecules were precipitated with BBM.1 (lane 5). In the immunoprecipitation with HA and W6/32, the β2m band was not detectable. In lane 6 (scK\(^b\) with HA), this is not expected, since the single chain construct probably does not associate with free β2m; in lanes 1, 2, and 4, this may be due to slow association of freshly synthesized β2m with class I heavy chains under these experimental conditions, for unknown reasons. Since the goal of the experiment was to detect both β2m and the heavy chain, it was a better choice to use the antibody BBM.1, which allows the visualization of (enough) radioactively labeled β2m throughout the experiment. Thus, I chose BBM.1 for the dissociation experiments.

### 3.3.5. HA–K\(^b\) molecules do not colocalize with LAMP1 after four hours of internalization

In Figure 3.5A, B, it was observed that suboptimally loaded dimers and peptide-bound HA-K\(^b\) molecules partially colocalize with early endosomes labeled with the early endosomal marker protein EEA1 as well as with some unidentified punctate structures. It is possible that these structures are late endosomes that contain the late endosome marker protein LAMP1. Thus, it is possible that HA-K\(^b\) molecules co-localize with LAMP1. To test this hypothesis, I
performed a microscopy experiment at 37 °C (as described in section 3.2.5) in the absence of lysosomal inhibitors, using the HA antibody against HA-K\(^b\) molecules and an antibody against LAMP1 as a marker of late endosomes. After one, two, and four hours, I did not see co-localization of HA-K\(^b\) molecules with LAMP1 at 37°C (Figure 3.14A). At other temperatures (experiments by Vaishnavi Venugopalan), there was also no co-localization of HA-K\(^b\) with LAMP1 (Figure 3.14B). Thus, in the time frame of the experiment, HA-K\(^b\) molecules either do not reach late endosomes, or are degraded very fast, such that they are not visible at any time point in late endosomes. For further experimentation, either a longer time frame should be examined (up to 10 hours, as described in section 2.3.2), or a lysosomal inhibitor such as ammonium chloride or chloroquine might be included during the incubation.

### 3.4. Endocytosis of H 2D\(^b\)

#### 3.4.1. HA-D\(^b\)-GFP molecules transiently transfected into STF1 cells are rapidly endocytosed in the absence and in the presence of peptides

To investigate whether for a different MHC class I allotype, my observations would still be correct, I decided to study H-2D\(^b\), another murine MHC class I allotype that is expressed in H-2\(^b\) cells and well-studied in the Springer laboratory. I transiently transfected STF1 cells with a construct encoding the fusion protein HA-D\(^b\)-GFP, performed a BFA decay experiment in the presence (10 \(\mu\)M) or absence of the H-2D\(^b\)-binding peptide FAPGNYPAL (FL9) [1],
and stained with conformation-specific (B22.249) or conformation-independent (HA) antibodies (Figure 3.15).

At time point zero, there was a dose-dependent expression of HA-Dβ-GFP molecules at the cell surface, but above a certain level of protein expression, the cell surface population of
Figure 3.15 HA-D^2-GFP molecules transiently transfected into STF1 cells are rapidly endocytosed in the absence of peptides, but slower on their presence

A. STF1 cells were transiently transfected with a construct coding for the fusion protein HA-Kb-GFP. Four hours after transfection, cells were incubated overnight at 25 °C. Next day, a BFA decay assay at 37 °C was performed in the presence or absence of the high-affinity peptide FL9 (10 µM).

B. Quantification of A. A gate was created on the GFP^+ cells, and the fluorescence intensity was normalized to the fluorescence at time point zero. Staining was done with B22.243 or HA. The figure is representative of three or more independent experiments.
HA-D<sup>b</sup>-GFP did not increase any more. In the absence of peptides, HA-D<sup>b</sup>-GFP was rapidly endocytosed, and there was no visible difference between the signals of the conformation-specific and the conformation-independent antibodies. In the presence of peptide, cell surface levels of HA-D<sup>b</sup>-GFP molecules remained entirely stable for two hours, but decreased rapidly afterwards, as measured with both B22 and HA.

Since the peptide FL9 has a high affinity for H-2D<sup>b</sup> (IC<sub>50</sub> = 22 nM in a peptide competition assay [1]), I expected to see a longer lifetime at the cell surface in the BFA decay experiment, just as I saw for H-2K<sup>b</sup>/SL8. Since this was not the case, I decided to repeat this experiment in the cell line where such experiments have often been done, namely RMA-S [2-5].

### 3.4.2. Endogenous H-2D<sup>b</sup> molecules in RMA-S cells are rapidly endocytosed in the absence of peptides but not in their presence

Since in STF1 cells, the FL9 peptide does not prevent the endocytosis of HA-D<sup>b</sup>-GFP molecules for more than two hours (Figure 3.15), I decided to repeat the experiment in RMA-S cells. Thus, I performed a BFA decay assay in RMA-S cells in the presence or absence of the peptide FL9 (10 µM), and then I stained with B22.249. As expected, in the absence of peptide, H-2D<sup>b</sup> molecules are rapidly endocytosed, but in the presence of FL9, the endocytosis of H-2D<sup>b</sup> is significantly slowed down (Figure 3.16). Therefore, it is clear that the peptide FL9 does prevent the endocytosis of H-2D<sup>b</sup> in RMA-S cells.

In general, the results obtained in the STF1 cells are not similar to those obtained in RMA-S cells. Therefore, the combination of STF1 cells and the construct HA-D<sup>b</sup>-GFP should not be used for further studies until the reason of the difference is identified. Since the construct was fully sequenced, mutations in the construct are not likely to be the case of the discrepancy between the results in RMA-S cells and STF1 cells. It is still possible that in the case of H-2D<sup>b</sup>, the addition of tags such as the HA and the GFP tag have an influence on its endocytosis phenotype. Comparison between constructs without tags might be an alternative for the study of H-2D<sup>b</sup> in STF1 cells.

### 3.5. Endocytosis of CD55

CD55, also known as the Decay Acceleration Factor (DAF), is involved in the regulation of the complement system at the cell surface [6]. Interestingly, CD55 is internalized via Arf6-mediated endocytosis, like class I, and it co-localizes with human MHC class I in the endocytic pathway [7]. At 25 °C, FHC can be endocytosed, but not sub-optimally loaded
It is not known whether other proteins that follow the Arf6-mediated internalization pathway can be endocytosed at 25 °C. Thus, it is interesting to know whether an additional protein that follows the same internalization pathway as MHC class I can be endocytosed at 25 °C.

Figure 3.16 Endogenous H-2D\(^{b}\) molecules in RMA-S cells are endocytosed fast in the absence of peptides, but not in their presence

RMA-S cells were incubated overnight at 25 °C. Next day, a BFA decay assay at 37 °C was performed in the presence or absence of the high-affinity peptide FL9 (10 µM). Staining was done with B22.243. The figure is from a single experiment done in triplicate.
To answer that question, I first wanted to identify whether CD55 is present at the surface of STF1/K\textsuperscript{b} cells, and whether it can be accumulated at 25 °C like H-2K\textsuperscript{b}. When I stained the cells and performed flow cytometry, it was evident that at 25 °C, there was less CD55 at the cell surface of STF1/K\textsuperscript{b} cells at steady state than at 37 °C. When I evaluated the endocytosis rate of CD55 in a BFA decay experiment, the endocytosis of CD55 was very slow already at 37 °C. At 25 °C, the cell surface level of CD55 was almost constant after four hours of treatment with BFA (Figure 3.17).

Overall, the endocytosis of CD55 at 25 °C is similar to the endocytosis of H-2K\textsuperscript{b} suboptimally loaded dimers at 25 °C. Since the goal of this experiment was to show that a protein other than MHC class I can be endocytosed at 25 °C, CD55 cannot be used as an example. I later used FHC for that purpose (Figure 3.2D).

3.6. Role of selected Rab proteins in the endocytosis of HA-K\textsuperscript{b}

3.6.1. Rab11

Rab11 is considered a marker of recycling endosomes (details in section 2.2.6). If recycling of any of the forms of HA-K\textsuperscript{b} molecules is mediated by Rab11, then the disruption of its function or its overexpression in very large amounts might interfere with the recycling of HA-K\textsuperscript{b}. This may be visualized in a BFA decay experiment, where a GFP fusion of Rab11 is transiently transfected into cells that express stably HA-K\textsuperscript{b} molecules. For a GTPase that supports recycling of class I from endosomes, I expect that its loss of function, for example,
Figure 3.18 Overexpression of GFP-Rab11WT apparently does not affect the endocytosis of HA-Kb molecules in STF1/Kb cells. 

A. STF1/Kb cells were transiently transfected with a construct coding for the fusion protein GFP-Rab11WT. 24 hours after transfection, cells were incubated overnight at 25 °C. Next day, a BFA decay assay at 37 °C was performed in the presence or absence of the high-affinity peptide SL8 (10 µM). Staining was done with Y3 or HA. B. Quantification of A. The figure represents a single experiment. U = untransfected; T = transfected. This experiment was done by Mohammed Al-Balushi.

brought about by the overexpression of a dominant negative mutant, leads to rapid
endocytosis of peptide-bound class I at 37 °C and of peptide-bound and suboptimally loaded class I at 25 °C. For a GTPase that mediates class I sorting into deeper endocytic compartments towards lysosomal degradation, I expect that its loss of function leads to a decrease in endocytosis of suboptimally loaded dimers at 37 °C.

To test this hypothesis, I created a plasmid that encodes the fusion protein GFP-Rab11WT. The plasmid was transfected into STF1/Kb cells; a day after transfection, the cells were incubated overnight at 25°C, and next day, a BFA decay assay was performed at 37 °C, in the presence or absence of SL8 peptide, and the staining was done with Y3 and HA (Figure 3.18; the experiment was done by Mohammed Al-Balushi).

Clearly, the transfection efficiencies were not very high (Figure 3.18, “T”). Still, in the cells that were transfected, the cell surface expression of HA-Kb molecules did not seem different from that in untransfected cells. Thus, at the observed protein expression level, GFP-Rab11WT does not alter the endocytosis of any of the forms of HA-Kb molecules.

Since the transfected vector expressed the WT version of the protein, it might be necessary to express a constitutively active or a dominant negative mutant that disrupts directly the function of Rab11. Rab11(S25N) is a dominant negative mutant of Rab11 (details in section 2.2.6 of the introduction). If the amount of Rab11(S25N) in the cells is enough to compete with the endogenous Rab11, it is conceivable that recycling of any of the forms of HA-Kb molecules might be affected.

To test this hypothesis, a similar experiment to that done with GFP-Rab11WT was performed. Again, as observed with GFP-Rab11WT, the transfection efficiency was not very high (Figure 3.19, “T”), and in the cells that were transfected, the cell surface expression of HA-Kb molecules did not seem different from that in untransfected cells (Figure 3.19) (Experiment by Mohammed Al-Balushi). Thus, as in the case of GFP-Rab11WT, at the protein expression level observed, GFP-Rab11(S25N) does not alter the endocytosis of any of the forms of HA-Kb molecules. As the previous experiment, this experiment is not conclusive. The times and protein expression levels have to be optimized.

Recycling via Rab11 might occur only in the first stages of endocytosis. Therefore, to evaluate the effect of recycling via Rab11, further BFA decay experiments should be done in shorter time intervals, such as every ten minutes up to one hour. An alternative approach
 would be to perform the Rab11 overexpression experiment at 25 °C, since at this temperature,
most HA-K\(^b\) molecules are at the cell surface, although they can reach the interior of the cell (Figure 3.5A) The rationale is that if Rab11 plays a role in the recycling of HA-K\(^b\), the internalized molecules might not recycle to the surface at 25 °C because Rab11 is inhibited.

### 3.6.2. Rab5

Rab5 is small GTPase that is characteristic of classic early endosomes (see section 2.2.6), and the respective dominant negative mutant is Rab5(S34N). If sorting from the early endosomes affects the cell surface expression of HA-K\(^b\) molecules, the overexpression of Rab5 or Rab5(S34N) might alter this process. Analogous to the experiments with Rab11, a variation in the endocytosis rate due to disruption of the function of Rab5 might be visualized in a BFA decay experiment.

To follow that hypothesis, STF1/K\(^b\) cells were transiently transfected with a construct that encodes the fusion proteins GFP-Rab5 WT or GFP-Rab5(S34N). A day after transfection, cells were incubated overnight at 25°C, and the next day, a BFA decay assay was performed. The time frame of the BFA decay was reduced to one hour, in intervals of 15 minutes, and the staining was done with anti-HA antibody (experiment by Vaishnavi Venugopalan).

This time, the transfection efficiency and the transfection levels were higher than those of Rab11WT and Rab11(S25N). At 37°C, the steady state cell surface expression of HA-K\(^b\) molecules did not change compared to the untransfected cells (Figure 3.20A). At 25 °C, in the cells that expressed the highest amount of total Rab5WT (R5 to R7), there were fewer HA-K\(^b\) molecules at the cell surface than in the cells with lower Rab5WT expression (Figure 3.20B). This was also true in the case of the cells transfected with Rab5(S34N). The endocytosis of HA-K\(^b\) molecules was independent of the expression level of either Rab5 protein (Figure 3.20C, D). Importantly, in this experiment, after 30 minutes, the cell surface expression of HA-K\(^b\) molecules in untransfected cells was already very low (Figure 3.20C, D).

Apparently, the overexpression of Rab5WT or mutant affects the steady-state levels of HA-K\(^b\) molecules at 25°C. However, WT and mutant have very similar effects. The low levels of HA-K\(^b\) molecules at the cell surface could be due simply to overexpression of the Rab proteins, rather than to the loss of a specific function of Rab5. As for Rab11, further experiments should be done to continue with this investigation.
Figure 3.20 Overexpression of GFP-Rab5WT and GFP-Rab5 (S34N) apparently do not increase the endocytosis of HA-Kb molecules in STF1/Kb cells

STF1/Kb cells were transiently transfected with a construct coding for the fusion proteins GFP-Rab5 WT or GFP-Rab5S34N. A. 24 hours after transfection, the steady state cell surface expression of HA-Kb molecules at 37 °C was analyzed by flow cytometry. B. Quantification of the steady state levels at 37 °C and 25°C. C. 24 hours after transfection, cells were incubated overnight at 25 °C. Next day, a BFA decay assay at 37 °C was performed. Staining was done with HA. D. Quantification of the BFA decay experiment after transfection with Rab 5WT. E. Quantification of the BFA decay experiment after transfection with Rab 5S34N. U = untransfected; R1 to R6: regions 1 to 6. The figure represents a single experiment. This experiment was done by Vaishnavi Venugopalan.
3.7. Additional references


4. Perspectives in MHC class I endocytosis

In this work, I have shown the decisive role that \( \beta_2 \)m has in preventing the endocytic destruction of the murine MHC class I molecule H-2K\(^b\). While this conclusion is a step in the elucidation of the molecular mechanisms that regulate the endocytosis of MHC class I molecules, it is evident that many factors and processes remain to be discovered. Considering the role of \( \beta_2 \)m in MHC class I endocytosis, there are at least three major questions that can be followed up:

**Universality:** is the dissociation of \( \beta_2 \)m from the heavy chain a universal trigger for the endocytic destruction of all (or the majority of) MHC class I molecules?

**Endosomes:** in which endosome does \( \beta_2 \)m dissociate from the HC?

**Signals:** is there any “destruction signal” exposed upon dissociation of \( \beta_2 \)m from the heavy chain?

In the following, I suggest some experimental ways to solve these and subsequent questions. Finally, I will highlight some novel techniques and experimental methods that are needed to solve the major questions in the field of endocytosis.

4.1. Universality

4.1.1. Endocytosis rate and \( \beta_2 \)m dissociation

To find out whether \( \beta_2 \)m association can protect all MHC class I molecules from endocytic destruction, two parameters need to be measured for several MHC class I molecules other than H-2K\(^b\): the endocytosis rate and the dissociation rate of \( \beta_2 \)m from the heavy chain. Since there are differences in some measured dissociation rates \([1, 2]\) I predict that there will be a correlation between these two parameters: the faster the dissociation rate, the faster the endocytosis of the MHC class I complex, as is the case for H-2K\(^b\) at different temperatures. This will show that the dissociation of \( \beta_2 \)m universally governs the endocytic destruction of MHC class I molecules. Ideally, one would like to analyze these two parameters in cells. For this, it is necessary to select a pool of well-studied MHC class I heavy chains (such as HLA-A*02:01, HLA-B*44:02, HLA-B*44:05, HLA-B*27:05, HLA-B*27:09, HLA-B*57:01, HLA-B*35:01, and HLA-B*35:03), and create HA-tagged variants of each of
them. Then, TAP-deficient cells such as STF1 can be transduced with a construct coding for
the respective HLA proteins, always providing excess β2m by means of the 2A sequence. Finally, the experiments done in this work can be repeated and compared: BFA decays, detection with anti-HA antibody and radioactive dissociation assays at 37 °C, following the disappearance of the HA band by densitometry, as in Figure 3.3C.

The study could be complemented with microscopy analyses, as done in this work, to identify whether the arrival of different MHC class I allotypes at a particular endosome, for example at EEA1+ early endosomes, occurs at the same time or at different times. The microscopic images can be quantified manually (using the JACoP plugin of Image J), or with a software that determines the colocalization automatically through a series of images.

4.1.2. Cell types and cell lines

A more challenging question is whether there are significant differences in the endocytic rate and endocytic mechanisms of MHC class I molecules between immortalized cell lines, primary cells, and professional antigen presenting cells (pAPCs). To mention a single example, most of the knowledge about the cellular mechanisms of internalization and recycling of MHC class I molecules available so far has been generated with HeLa cells, by means of the antibody mediated internalization assay, with a single antibody, W6/32 [3]. Thus, it has not been corroborated what proteins are involved in the endocytosis of MHC class I molecules in primary cells.

Consequently, it is desirable to obtain primary cells of different tissues and perform biochemical, microscopy, or flow cytometry experiments with them. Since this is might be a challenging task in certain cell types, the evaluation of a single MHC class I allotype in several different primary cell types might be sufficient to provide mechanistic evidence comparable to the data on HeLa cells.

It will be particularly important to address whether the mechanisms described so far are also valid for professional pAPCs. Indeed, in this work I have observed that there are very few FHC at the cell surface at steady state, as opposed to what other researchers have found using different experimental systems and readouts (in particular in the case of professional pAPCs, see Table 2.1). One potential explanation is that in such cells, there are overall more MHC class I molecules present at the cell surface compared to other cell types, and as a result of that, there are more chances to form FHC at the surface, while the majority are still produced.
in the endosomes. It is also possible that in pAPCs, the mechanism is different, and the FHC are mainly generated at the cell surface.

Among the pAPCs, dendritic cells are particularly interesting. The source of the MHC class I molecules that crosspresent antigens has been postulated to be the ER through its fusion with phagosomes that carry the phagocytosed antigens [4]. This mechanism has been postulated to deliver components of the PLC that help to load peptides onto the MHC class I molecule [5]. From my work and that of others [6, 7], it appears that the source of MHC class I molecules that cross present peptides might also be the cell surface. This is supported by the observation that IRAP localizes to a Rab14+ endosome (see section 2.2.6), which in turn, co-localizes with EEA1; thus, Rab14 endosomes should be accessible to suboptimally loaded dimers in STF1/Kb cells (Figure 3.5A). In this hypothesis, the dissociation of β2m from the HC in an endocytic compartment might play an important role in the process of crosspresentation. To test this hypothesis, one might do a basic characterization of endocytosis rates in a dendritic cell line such as MutuDC, THP-1, or KG-1 after differentiation with cytokines [8-10]. Then, a major study with dendritic cells isolated from healthy patients or from mice should be performed.

In polarized cells, it will be important to address whether there is a difference between apical and basal internalization of MHC class I molecules and the respective recycling and lysosomal degradation. These studies can be started in polarized cell lines such as Caco-2 [11] by evaluating the cell surface levels of MHC class I, identifying the allotypes, and performing the standard experiments described above. A stringent microscopy characterization should be done to identify differences between both surfaces.

4.2. Endosomes

4.2.1. Characterization of the endosome in which β2m and the HC dissociate

One of the major unknowns that remain is where the dissociation of β2m from the heavy chain takes place inside the cell. The evidence presented here suggests that H-2Kb dimers have access to EEA1-positive early endosomes. The next experiment – which has not yet been done in STF1/Kb cells – is whether dimers also reach late endosomes. My prediction is that dimers will dissociate before, or as reach the highly acidic environment of late endosomes or lysosomes, since the dissociation of β2m from the HC is pH-dependent [12], with β2m dissociating at pH values below 5.5 [13].
Two published microscopy approaches have made use of the addition of fluorescently labelled β2m as a marker for MHC class I endocytosis [12, 14]. In these experiments, the exogenous β2m might bind not only to MHC class I but also to some other cell surface proteins. Thus, a co-localization experiment with fluorescently labelled β2m and different endocytic markers will mainly show the fate of β2m. The essential requirement for an intracellular dissociation experiment between two proteins is that upon dissociation, the two proteins can be detected independently. An initial experiment could be as follows: if there are two fluorescently labelled Fab fragments, one against HA (green), the other one against β2m (red), then an antibody-mediated internalization assay could be done in STF1/Kb cells. The antibodies will be titrated such that upon incubation of the cells with the antibodies, the signal yields a good co-localization, as quantified by Pearson’s coefficient. Importantly, some of the endogenous MHC class I molecules will also be labeled by the anti-β2m antibody. Once this is established, the internalization step can be done at 37 °C, for up to 20 minutes, the time in which dimers were observed in early endosomes. After 20 minutes, it is essential to do a brief acidic wash to remove any β2m that is still bound to the surface and thus to look only at internalized material. Then, the individual fate of both proteins can in principle be detected at different time points.

If this experiment works, i.e., the majority of the complexes are yellow at the beginning, and then they separate into green and red, the next question to be solved is the marker of the endosome. Co-localization experiments are required, either with GFP fusions of some Rab proteins (Arf6, EHD1, Rab11, and Rab22) or with antibodies, to detect whether β2m and FHC localize to the compartments that are marked by these GTPases. It will be interesting to see by microscopy or flow cytometry whether there is (fast or slow) recycling of H-2Kb dimers, something that can be in principle detected in case there is co-localization found with Rab4 or Rab11 (markers of fast and slow recycling, as described in section 2.2.6), respectively.

Another way to determine the identity of endosomes that are accessible to different forms of class I is a classical membrane fractionation assay such as the one performed by Gromme [13], in which each organelle is distinguished by a marker protein, for example the transferrin receptor for recycling endosomes. In isolated organelles, different forms of class I are maintained, as the Springer group have shown, and they can be detected with conformation-dependent antibodies and peptide addition [15, 16].
In addition to protein markers, it will also be important to know what the pH of the endosome is where β2m and the HC dissociate. The most intuitive way to perform such an experiment is to use pH-sensitive fluorescent dyes [17] and live cell imaging. In theory, it is possible that such a dye can be coupled to a carrier, for example, a polyelectrolyte microcapsule [18], such that they can be added to the cells to phagocytose, and eventually cross the entire endocytic pathway. The problem with this approach is that the phagocytosis of capsules might have a completely different endocytosis mechanism and, thus, might never cross through the endosome where the dissociation occurs. An alternative approach can be the use of pHfluorin2 constructs, which are variants of GFP that are sensitive to pH changes [19]. In this experimental method, an MHC class I heavy chain can be tagged with a pHfluorin2 tag at its amino terminus, transfected transiently into TAP negative cells, and followed by confocal microscopy. The main problem with this approach is the background of different organelles, such as the ER, where most of the expressed HC will be present. Still, this approach might be optimized.

Clearly, all these approaches are technically demanding and require the development of new techniques.

4.2.2. Functionality of the “dissociation endosome”

The physiological relevance of the dissociation compartment makes it attractive to identify. This endosome might be, for example, one of the key factors that differentiate pAPCs from fibroblasts in terms of antigen presentation, since it has been claimed that in immature dendritic cells, the pH of lysosomal organelles is elevated, enabling the persistence of peptides generated from endocytosed material [20, 21]. In the same way, a change in the pH of endocytic organelles in any type of cell will alter the dissociation rate of β2m from a MHC class I HC, which in turn might change the repertoire of antigens presented at the cell surface. Thus, the molecular assays presented in section 4.1.1 might become of great help to identify why in certain cells there is more efficient antigen presentation than in others.

In the particular case of crosspresentation, it would be very interesting to ask whether there is peptide binding or peptide editing in such an endosome. Based on the observations that small peptides can stabilize MHC class I molecules at the cell surface [22], and that such peptides can promote the exchange of peptides loaded onto the MHC class I molecule [23], one can speculate that an endosome may contain such small peptides or similar molecules that prevent β2m from dissociating from the HC. If that were true, this will suggest a new hypothesis in the
mechanism of crosspresentation: in this imaginary mechanism, it is not necessary to have an active transporter of peptides (such as TAP) or a chaperone that edits peptides (such as tapasin) at the level of the endosome, because the MHC class I molecules will come from the cell surface, and the peptides will come from degradation of other (perhaps extracellular) proteins inside the endosome. Since peptide-bound MHC class I molecules are internalized and can recycle to the cell surface, there is a possibility that peptides are exchanged in this endosome, and then class I molecules are returned to the cell surface with the new peptides via the endocytic recycling pathway.

4.2.3. Is $\beta_2m$ liberated to the blood via exosomes?

Dissociation of $\beta_2m$ from the HC leads to lysosomal degradation of the two proteins. However, $\beta_2m$ is found in the blood at high concentration (see section 5.1.1). If the majority of FHC are generated inside the cell, then the majority of the $\beta_2m$ in the blood should not come from the dissociation of $\beta_2m$ from the HC at the cell surface. There are at least four alternative possibilities to explain the origin of $\beta_2m$ in the bloodstream: first, $\beta_2m$ is secreted constitutively and released directly into the bloodstream, independent of MHC class I; second, $\beta_2m$ is synthesized and bound to a molecule other than class I and dissociates from it at the cell surface; third, $\beta_2m$ dissociates from the HC in endosomes and is recycled to the surface and secreted independent of any other protein; and fourth, $\beta_2m$ dissociates from the HC in endosomes, binds to the extracellular domain of a membrane protein that is present in the luminal vesicles of multivesicular bodies, and then is released to the cell exterior at the surface of an exosome. Indeed, there is evidence that B cell and dendritic cell exosomes contain at their surface MHC class I and MHC class II molecules that are partially functional [24]. Assuming that a good portion of the synthesized $\beta_2m$ in cells is bound to MHC class I molecules, it is not totally unlikely that a major portion of $\beta_2m$ is actually released to the outside of the cell via exosomes.

Taken together, the characterization of the dissociation endosome does not appear to be an easy task. It will require major technical advances and substantial thought about the entry experiments. Still, it is a fundamental question that can elucidate more than a single mechanism in both antigen presentation and endocytosis.
4.3. Signals

Several reports indicate the requirement of various post-translational modifications for the endocytosis of MHC class I molecules. Some post-translational modifications such as phosphorylation or ubiquitination have been reported as necessary, but not sufficient [25, 26], or they have been reported to be essential, but only in one particular case [27].

4.3.1. Ubiquitination

It has been described that when the ubiquitin ligases MARCH IV and MARCH IX are present in the cell, the lysines present in the cytosolic tail are ubiquitinated and the MHC class I complex is targeted for lysosomal degradation [27]. The same phenomenon has been observed in the presence of the viral ubiquitin ligases E3 and E5 [28]. In this study, the role of ubiquitin mutants in the inhibition of endocytosis in STF1/Kb cells was evaluated, but no effect was detected (Corinna Kulicke, unpublished BSc thesis, 2013). In the literature, there are no reports to my knowledge that show a correlation between ubiquitination and endocytosis in cells that have not been infected by a virus or where MARCH IV and MARCH IX are absent.

Overall, if ubiquitination is a signal for destruction of MHC class I molecules, it does not seem to be a general characteristic. It is possible that ubiquitination of MHC class I molecules plays a role only in some tissues, or at a very late stage such as the uptake into the luminal vesicles of multivesicular bodies, as shown for MHC class II [29]. Alternatively, ubiquitination may play a role at a stage different than the internalization or recycling, for example, upstream of a signaling cascade.

4.3.2. Phosphorylation

Phosphorylation of tyrosine residues in the cytosolic tail of different MHC class I molecules has been also described as a requirement for endocytosis [25, 26]. It is possible that phosphorylation of a tyrosine residue located at the cytosolic tail does not occur at the cell surface, but in an endosomal compartment. In support of this hypothesis, there is a study that found that the kinase Yes (a tyrosine kinase member of the Src family of kinases) phosphorylates the epidermal growth factor receptor (EGFR) in endosomal fractions of polarized cells [30]. In that study, the phosphorylation of EGFR at the apical recycling endosome promoted the transcytosis of the polymeric immunoglobulin A (pIgA) -polymeric immunoglobulin receptor (pIgR) complex towards the apical membrane. In the case of MHC class I, it might be possible that such a signal exists, by means of the kinase Yes. The
experimental method to test whether there are kinases that phosphorylate a residue at the cytosolic tail of MHC class I molecules requires the enrichment of endosomal fractions (as described in section 4.2.1), and the further in vitro kinase assay, as described in [30].

Alternatively, if phosphorylation and/or ubiquitination play a role in the endocytosis of MHC class I molecules, it is more likely to play a role upstream of the MHC class I molecule itself, as a result of a signaling cascade initiated by an unknown protein.

4.3.3. Other proteins

It has not been ruled out that there is a novel unknown protein that surveys the β2m binding status of MHC class I molecules. Perhaps upon dissociation of β2m, an unknown protein binds to the FHC and delivers it to lysosomal degradation. To find such a protein, Donaldson and co-workers [31] arrested the internalization of MHC class I molecules with the use of a constitutively active mutant of Arf6 and then isolated the vesicles in which MHC class I and other proteins were present. Using this approach, one might immunoprecipitate β2m or the MHC class I heavy chain in a mild detergent such as digitonin, and perform proteomic analyses.

An alternative to finding proteins involved in the endocytosis of MHC class I molecules might be a forward genetic screen using near-haploid cells such as KBM7 or HAP1 [32]. Haploid screens were originally developed to identify host factors used by pathogens, but in principle, they can be applied to solve any other molecular question. One selects a gene-trap vector [33] and transduces it into the haploid cell line to generate a panel of mutants. The subsequent behavior of these mutants, for example their viability after application of a toxin, is then evaluated, for example, with the flow cytometer. Then, in the cells whose phenotype is altered, the site of the insertion of the gene-trap transposon is determined by PCR and sequencing.

In the case of MHC class I, one might use a haploid screen to discover mutants that accelerate class I disappearance from the cell surface, such as those that inhibit the recycling of class I trimers to the cell surface. Following the generation of mutants, anterograde transport is inhibited with BFA, peptide is added to convert all class I molecules into trimers, and after some time, all cells that still carry class I molecules at the cell surface are either sorted out by FACS, or killed with anti-class I antibody and complement. The remaining cells are then
grown out and characterized. Similar screens are possible for mutants that have slow class I removal kinetics.

4.3.4. Lipids

It was recently discovered that the enzyme diacylglycerol kinase is required for the formation of tubular recycling endosomes as well as the recycling of MHC class I molecules in HeLa cells [34]. This observation enables the study of the role of lipids in the endocytosis of MHC class I molecules. The entry experiment will be to knock down the enzyme diacylglycerol kinase by means of siRNA or with the CRISPR/CAS system. Then, one would perform flow cytometry experiments, detecting dimers and peptide-bound MHC class I molecules with the respective antibodies (for example, 25-D1.16 for K\(^b\)-SL8, and Y3 for dimers). If the enzyme is knocked down entirely, it is possible that recycling stops completely, or that only dimers are not detected at the cell surface. The recycling of the TfR should be unaltered. As a complementary approach, it would be interesting to express GFP-tagged proteins or protein domains that have affinity or selectivity to different phospholipids [35] and perform co-localization experiments between the different forms of MHC class I.

4.4. Techniques

In the field of endocytosis, progress is limited by the availability of suitable assays. Some potential future developments are listed in the following.

*In vitro* compartment reconstitution or fusion assays in which the role of the different Rab proteins and other endocytic regulators can be evaluated have been described for the study of clathrin-mediated endocytosis [36]. One such experimental system has been created to reconstitute recycling vesicles [37], another to reconstitute clathrin-independent endocytosis in semi-intact polarized cells [38]. While these systems might provide a clean biochemical environment, their establishment is tedious and usually takes a long time.

An interesting assay developed recently to study the secretory pathway, called the 'retention using selective hooks' (RUSH) system [39], could be implemented in the study of endocytosis. The assay makes use of two fusion proteins, the “hook”, which is fused to a modified streptavidin molecule and modified with an ER retention signal, and the “reporter”, which is fused to a streptavidin-binding peptide. Thus, the two proteins interact, and the reporter is retained in the ER. Upon addition of biotin, the interaction is disrupted, and the reporter protein is released to its cellular destination. In principle, with a similar system, it
might be possible to observe the entire process of secretion and endocytosis of a protein upon release from the hook. It might be more interesting if only the endocytosis part could be followed; this might be achieved with a hook that is firmly anchored at the cell surface. Such experiments require a live cell imaging microscope.

Another interesting idea comes from the combination of two experimental systems that are already available in the Springer group. One can print an antibody against a particular MHC class I allotype onto a flat glass surface [40] and then grow cells on that surface. If the cells express an MHC class I allotype fused to a fluorescent protein, the immobilization of this class I molecule at the cell surface – due to its binding to the antibody – can be visualized by microscopy (Cindy Kroll and S. Springer, unpublished). Then, high-affinity peptides could be added to the cells to prevent the endocytosis of MHC class I molecules. Addition of catalytic dipeptides to the cells [23] might lead to the release of the high-affinity peptides from the peptide-binding groove of the MHC class I molecule, loss of binding to the conformation-specific printed antibody, and subsequent concerted endocytosis of all class I molecules from the cell surface. The entire process might be examined by life cell imaging, and the fate of the MHC class I molecules through the endocytic pathway might be followed.

Whenever antibody-labeled or GFP-tagged proteins are followed by microscopy, the question of what is actually being followed often comes up. This is the main drawback of antibody-mediated internalization assays (since one observes the dye on the antibody, not the target protein), or the experimentation with GFP-tagged proteins (since GFP may be proteolyzed from the target protein in the endocytic pathway). The best way to avoid these questions might be to develop fluorescent probes that can be directly covalently bound to the protein of interest, for example SNAP tags or sortase tagging [41-43].

The use of drugs that inhibit the different steps of endocytosis is still a major area of investigation. An example is the case of the drug Pitstop, which is supposed to inhibit clathrin dependent and independent endocytosis [44, 45]. I could not see any inhibition of MHC class I endocytosis (not shown). In the meantime, Pitstop was shown to inhibit endocytosis in a different way as first described [45, 46], and I find it very difficult to trust such drugs and others such as such as chloroquine, primaquine, and concanamycin that may have all kinds of non-specific side effects [47]. Substantial work is required to find an optimal and specific inhibitor of endocytosis.
Finally, the study of MHC class I endocytosis might lead to the generation of new high-throughput assays that use the concepts of endocytosis and receptor-ligand interaction. This kind of assay would be helpful to address questions about receptor-ligand interaction at the surface of cells, as opposed to approaches such as surface plasmon resonance, in which only the receptor and the ligands are considered. An imaginary setup could be as follows: if it were possible to produce a microarray of ligands bound to a surface, and then seed cells that express the respective receptor (fluorescently tagged) on top of them, it could then be possible to follow by microscopy (or a plate reader) the places in which the cells are more (or less) fluorescent, according to the receptor. In the case of MHC class I, the presence of a high-affinity peptide will rescue them from endocytosis. Thus, the readout will be an increase in the fluorescence intensity at the cell surface. Clearly, there are several controls that may not make possible the feasibility of such a system, but the general idea is that the knowledge on the endocytosis of a receptor and the interaction with the ligands can be exploited and turned into a product.
4.5. References

5. In vitro studies of beta-2 microglobulin in solution

5.1. Introduction

Generally, proteins need to be folded to be functional (although “natively unfolded” proteins do exist [1]). When incorrectly folded proteins fail to be degraded by the cells, they accumulate, and in some cases, this accumulation will lead to disease. Prominent cases of protein unfolding that lead to disease are the Alzheimer and Huntington diseases, where the underlying principle is the formation of protein aggregates (amyloids) that accumulate in the cell [2]. In order to elucidate how such diseases occur, it is necessary to study protein folding and protein structure.

To learn about protein folding, conformation, and protein structure in general, purified proteins can be studied *in vitro* by several methods. Typically, proteins are analyzed by X-ray diffraction, which after processing yields a crystal structure. In the crystal structure, proteins are rigid, as opposed to what happens in living cells, where proteins are dynamic structures, that is, they move. Despite the amount of information that can be obtained from a crystal structure, it does not provide information about how proteins move. Thus, in addition to the crystal structure, it is necessary to find alternative methods and parameters to describe protein mobility.

Thermodynamic parameters such as ΔH (enthalpy), ΔG (free energy), or ΔS (entropy) of folding - which to some extent indicate protein mobility – have been commonly measured by calorimetric methods, such as differential scanning calorimetry (DSC)\(^2\) or isothermal titration calorimetry (ITC). These methods are sometimes accompanied by the use of spectroscopic methods, such as circular dichroism (CD) or tryptophan fluorescence. With the former methods, it is possible to determine changes in the secondary structure of a protein, as well as the loss of structure – unfolding – upon temperature increase. There are several examples where spectroscopic methods have been used in addition to thermal methods to identify the conformational properties of several proteins such as BSA [3], lysozyme [4, 5], hexokinase [6], and the prion protein Prp\(^{sc}\) [7].

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\(^2\) Abbreviations: \(\beta_2m\): beta-2 microglobulin; CD: circular dichroism; DSC: differential scanning calorimetry; DTT: dithiothreitol; h\(\beta_2m\): human beta-2 microglobulin; Ig: immunoglobulin; ITC: isothermal titration calorimetry; MHC class I: major histocompatibility complex class I; NMR: nuclear magnetic resonance; TCEP: tris (2-carboxyethyl) phosphine; TDTF: thermal denaturation measured by tryptophan fluorescence. \(T_m\): denaturation temperature
Another protein whose folding and structure has been well characterized is beta-2 microglobulin (β₂m). In this study, β₂m will be used as a model protein to investigate protein mobility and flexibility. In the following section, a detailed description of β₂m, both as an individual protein and in complex with the major histocompatibility complex class I (MHC class I) heavy chain will be made.

5.1.1. β₂m: general aspects

Beta-2 microglobulin is a globular protein of nearly 13 kDa that is commonly known as the light chain of the MHC class I complex. It was discovered in 1968 in the urine of patients with chronic kidney dysfunction [8], and since then, it has become one of the three classical markers of kidney malfunction [9]. Beta-2 microglobulin is produced at 200 mg/day in adults and is catabolized only via the kidney [10]. In vivo, β₂m concentration in the blood is about 0.1 µM (ca. 1.1 µg/ml) [11]. In patients with critical kidney dysfunction undergoing dialysis, the plasma concentration of β₂m can rise significantly [10], which can lead to the formation of amyloid fibrils [12].

5.1.2. Structure and folding

The gene that encodes β₂m is on chromosome 15, outside the MHC. The translated protein has 99 residues and consists of a signal peptide that is cleaved upon translocation in the endoplasmic reticulum, followed by the mature, soluble protein. Beta-2 microglobulin belongs to the immunoglobulin (Ig) superfamily of proteins [13, 14], and it is widely conserved among mammals [15]. There are also natural mutant alleles of β₂m in humans [16] and mice [17].

Beta-2 microglobulin has seven antiparallel beta strands arranged in two sandwiched sheets that are connected by a buried disulfide bond (Figure 5.1) [14, 18]. The best-known interaction partner of β₂m is the heavy chain of MHC class I molecules [18]. Apart from MHC class I heavy chains, β₂m functionally interacts with a number of proteins such as APLP2 [19], CD1 [20], and the FcRn receptor [21].

The folding of β₂m proceeds via two different intermediates [22], and the reaction is rate-limited by the cis-trans isomerization of proline 32 [12]. The characterization of the folding of β₂m was possible because of some intrinsic properties of the protein. In the context of the present work, it is important to mention three of these properties. First, β₂m has two
tryptophan residues, W60 and W95. W95 is important for the stability of the folded state, whereas W60 is exposed to the solvent and important for binding to MHC class I [23].

Second, thermal stability investigation of β2m by DSC [24], CD [25], and thermal denaturation measured by tryptophan fluorescence (TDTF) [26, 27], has given a denaturation temperature (T_m) between 61 °C and 64 °C, depending on the method. Finally, between cysteine residues 35 and 84, there is a disulfide bond that is important for the stability of the protein. These two cysteines can also form intermolecular disulfide bonds in the context of the formation of amyloid fibrils [28-32].

Recent studies have demonstrated that β2m is conformationally flexible when it is not bound to the MHC class I heavy chain [15, 33, 34], particularly in the DE loop (Figure 5.1), which seems to be very important in the determination of the overall properties of native and partially folded β2m [11]. Supporting the notion of conformational flexibility within the DE loop, a study by Ricagno et al [35] showed that residues W60 and S57 (which are in the DE loop) from three different crystal structures populate distinct regions of the Ramachandran plot (i.e., assume different φ and ψ angles) depending on whether β2m is in complex with MHC class I or a monomer. This result indicates that β2m changes its conformation at the DE loop upon binding to the MHC class I heavy chain; in turn, molecular dynamics simulations also support the idea of conformational flexibility at the DE loop of β2m [36].

Figure 5.1 Structure of human β2m
The tryptophan residues 60 and 95 are highlighted in green. The DE loop is highlighted in maroon. The disulfide bond is highlighted in yellow. Crystal structure is from the protein data bank, accession number 1LDS.
The dissociation of $\beta_2m$ from the MHC class I heavy chain also increases its conformational dynamics, which leads to the creation of regions within $\beta_2m$ that are prone to form aggregates [37]. An example of increased conformational flexibility and escape of control from the cells is the natural $\beta_2m$ mutant D76N, which is more prone to form amyloid fibrils when released into the bloodstream [10]. This $\beta_2m$ mutant is more susceptible to protease destruction than the wild type, but still it is present at the cell surface bound to the heavy chain of HLA-A*02:01. The authors hypothesize that the mutant $\beta_2m$ can reach the cell surface despite its conformational flexibility because binding to the heavy chain reduces its flexibility and thus it can travel through the secretory pathway like a wild type $\beta_2m$ molecule; however, when it dissociates from the heavy chain, it has higher chances to form amyloids [34]. The authors called the MHC class I molecule the “Trojan horse” since it carries the otherwise conformationally unstable mutant $\beta_2m$ to the cell surface [34].

### 5.1.3. Binding to MHC class I

$\beta_2m$ binds non-covalently to MHC class I heavy chains [18]. It provides conformational stability to the heavy chain and strongly facilitates peptide binding and antigen presentation [14]. When the class I heavy chain is first synthesized in the ER, it is prone to unfolding and requires binding of the chaperone calnexin. Once the heavy chain associates with $\beta_2m$, calnexin is released [38]. Binding of $\beta_2m$ to the heavy chain is essential for efficient cell surface expression of all MHC class I molecules investigated so far [39].

Beta-2 microglobulin of one species can often bind to the MHC class I heavy chain of another species; for example, human $\beta_2m$ (h$\beta_2m$) can bind to murine MHC class I heavy chains. Indeed, H-2K$^d$, H-2D$^d$, and H-2L$^d$ are more conformationally stable when bound to h$\beta_2m$ than when bound to murine $\beta_2m$ [40, 41]. Additionally, the conformation-specific antibody W6/32 (directed against human MHC class I molecules) detects murine H-2D$^b$ heavy chains only if they are bound to the bovine $\beta_2m$, which is present in the fetal bovine serum [42].

In terms of binding affinities, it is important to highlight the work by Søren Buus and co-workers [43]. Radioactively labeled h$\beta_2m$ was incubated with purified H-2K$^k$ molecules (heavy chain + murine $\beta_2m$), and binding of radiolabeled h$\beta_2m$ to the heavy chain of H-2K$^k$ was measured after separation of the bound and unbound fractions by Sephadex G50 column chromatography. The assay was performed at various temperatures, namely 4 °C, 16 °C, 26 °C, and 37 °C. The binding of h$\beta_2m$ was rapid at 37 °C, and it was slower at lower temperatures. Importantly, even at 4 °C, binding was still detected. This binding reaction
presumably occurred after exchange of the pre-bound murine β₂m, although the authors did
not exclude the possibility that some free heavy chains were present in their preparation. The
authors calculated an association rate constant at 37 °C of 48 000 M⁻¹s⁻¹ assuming pseudo-first
order kinetics. Dissociation of hβ₂m from the heavy chain of H-2K^k molecules was also
temperature dependent, with fastest dissociation at 37 °C. The dissociation half-time at 37 °C
was one day (dissociation rate of 8 x 10⁻⁶ s⁻¹), and two days at 26 °C. In a more recent
experiment, the dissociation of hβ₂m from the heavy chain of HLA-A2 at 37 °C was measured
by hydrogen/deuterium exchange monitored by electrospray ionization mass spectrometry,
yielding a rate constant of 3.3 x 10⁻⁵ s⁻¹, approximately four times faster than the dissociation
of hβ₂m from H-2K^k [37].

5.1.4. Aim of this study

The aim of this study was to evaluate whether the conformational flexibility of β₂m at various
(and elevated, i.e., greater than 37 °C) temperatures can be measured indirectly by reduction
of its buried disulfide bond with a small molecule.

In the work described here, the accessibility of the buried disulfide bond of β₂m to the
reducing agent tris (2-carboxyethyl) phosphine (TCEP) at various temperatures was used as a
parameter to study its conformational flexibility in vitro, and for the potential comparison
with an analogous in silico simulation. Theoretically, if the rate constants of reduction are
deduced, one can correlate them with individual molecular collisions between the disulfide
bond and the reducing agent on a nanosecond time scale that were calculated from molecular
dynamics simulations. By this unique correlation, one could standardize molecular dynamics
simulations to the laboratory experiment.

5.2. Results

5.2.1. Establishment of the conditions to obtain monomeric β₂m in solution

In order to study conformational properties of β₂m by fluorescence spectroscopy, it is
necessary to have in solution a concentration of monomeric β₂m that is high enough to yield a
signal in the fluorimeter, but not too high, to avoid the formation of oligomers. Thus, I first
wished to establish conditions in which β₂m can be folded in vitro from urea-dissolved
bacterial inclusion bodies without formation of disulfide bonded oligomers.
For this, I performed two experiments. First, I folded 500 µg/ml hβ₂m in refolding buffer \[27\], pelleted the insoluble material by ultracentrifugation, and dialyzed the soluble material extensively against PBS. Finally, I took an aliquot of the preparation and separated it by reducing or non-reducing SDS-PAGE. Under these conditions, β₂m was partly present in the solution as disulfide-bonded oligomers (Figure 5.2A). Thus, under these conditions, it was not possible to investigate monomeric β₂m. In the second experiment, I decreased the concentration of folded β₂m from 500 µg/ml to 100 µg/ml and avoided the dialysis step. Under these conditions, the oligomers were no longer present (Figure 5.2B). The β₂m band migrated slightly faster in the non-reducing (NR) lane than in the reducing (R) lane (Figure 5.2B), indicating that the disulfide bond was correctly formed. Thus, I concluded that 100 µg/ml β₂m in refolding buffer are the conditions necessary to obtain β₂m in a monomeric state.

5.2.2. Denaturation of β₂m at room temperature requires a high concentration of acid or reducing agent.

For spectroscopic measurements, it is necessary to identify whether the monomeric preparation of β₂m yields a signal that can be detected by tryptophan fluorescence. I thus folded 100 µg/ml β₂m, and after ultracentrifugation, I collected the soluble material, diluted it in PBS, and recorded emission spectra with the excitation at 290 nm. The emission spectrum of untreated β₂m indicated that there was a detectable tryptophan fluorescence signal that was
strong enough for further measurements (Figure 5.3). Next, I asked whether the spectrum would change upon denaturation of β₂m. I followed the same procedure as above but treated a sample with 100 mM HCl and recorded the emission spectrum. In the presence of 100 mM HCl, the unfolded protein yielded a signal of 30% of the initial fluorescence intensity, with the same peak wavelength. I took this to be the baseline signal after denaturation.

Since β₂m has a buried disulfide bond, it is theoretically possible to reduce it by adding a reducing agent. Reduction with a reducing agent depends on the concentration of the reducing agent and on the conformation of the protein. Therefore, it is possible to investigate at what concentration of reducing agent β₂m will be reduced. Theoretically, the greater the flexibility of β₂m, the easier it will be for the reducing agent to gain access to the disulfide bond. Thus, this information can be used as a measure of the conformational flexibility of β₂m at room temperature.

To follow that hypothesis, I proceeded as above and treated the samples with 1 mM or 10 mM of the reducing agent Tris (2-carboxyethyl) phosphine TCEP [44] and then measured an emission spectrum. In the presence of 10 mM TCEP, the signal was reduced to 55%, whereas in the presence of 1 mM TCEP, there was almost no decrease in the fluorescence intensity (Figure 5.3). This means that at room temperature, the reduction of the disulfide bond of β₂m leads to a loss of structure, which, in turn, leads to partial quenching of the
tryptophan residue and which may, or may not, correspond to complete denaturation. In the presence of 10 mM TCEP, it is very likely that the disulfide bond was reduced, but there was some structure left. In the presence of 1 mM TCEP, the disulfide bond was not reduced. Overall, the concentration of TCEP necessary to reduce the disulfide bond of β2m at room temperature is ≥10 mM. Taken together, these results suggest that β2m at room temperature is not flexible enough to allow the access of TCEP (when present at a low concentration in solution, i.e., 1 mM) to the center of the protein, where the disulfide bond is.

5.2.3. β2m is conformationally flexible upon thermal denaturation and reduction of the disulfide bond

I next wished to test the hypothesis that at higher temperatures (greater than 37 °C), β2m is more flexible, such that its denaturation is possible with lower concentrations of TCEP than are needed at room temperature. If this hypothesis is true, TCEP in low concentration can access the disulfide bond of β2m and reduce it.

Data about folding and unfolding of β2m can be obtained by tryptophan fluorescence spectroscopy, since β2m has a tryptophan residue that is not exposed to the solvent when folded (section 5.1.2); upon exposure to the solvent (unfolding), the intrinsic fluorescence of W95 is quenched [23, 45]. The influence of the temperature can be evaluated by TDTF. In this method, β2m is exposed to thermal denaturation, and upon unfolding, W95 becomes exposed to the solvent. The temperature at which the protein unfolds can be detected as a transition point in the curve of temperature versus fluorescence intensity. This temperature is the so-called thermal denaturation temperature, and it is characteristic of each protein. The higher the temperature required to denature the protein, the more conformationally stable it is [27].

I thus folded 100 μg/ml β2m in refolding buffer. After ultracentrifugation, I diluted the soluble material into 3 parts of PBS and divided it into three aliquots. I treated the aliquots with 1 mM, 10 mM, or no TCEP. Immediately after addition of the TCEP, I recorded a TDTF curve to determine the Tm.

From the curves, I observed that the denaturation temperature of β2m in the absence of reducing agent was 62 °C, as reported in the literature (Figure 5.4). In the presence of 10 mM TCEP, there was no visible denaturation of β2m, suggesting that the protein was already unfolded at room temperature. Surprisingly, in the presence of 1 mM TCEP, the Tm
of $\beta_2m$ decreased to 54 °C, which means that with 1 mM TCEP, it is possible to reduce the disulfide bond of $\beta_2m$ already at 54 °C. In the absence of TCEP, the heat denaturation of $\beta_2m$ only begins at around 55 °C, and the $T_m$ is 62 °C (see above). The difference between the $T_m$ of 62 °C and 54 °C must be due to the reduced disulfide bond. Since the $T_m$ of $\beta_2m$ with 1 mM TCEP is 54 °C, this result suggests that at 54 °C, while $\beta_2m$ is still folded overall, its conformation is altered, or fluctuates, in such a way that even a small amount of reducing agent (1 mM) can access the buried disulfide bond.

Upon reduction of the disulfide bond, $\beta_2m$ rapidly denatures. This result supports the interpretation that conformational flexibility of $\beta_2m$ can be detected with the help of a small molecule reducing agent.

5.2.4. The accessibility of the disulfide bond of $\beta_2m$ to TCEP is temperature dependent.

Since the amount of TCEP required for denaturation decreased to 1 mM at 54 °C, I next wished to investigate whether it is possible to correlate the flexibility of $\beta_2m$ to the temperature by measuring the rates of reduction at various temperatures. From an experiment in which the concentration of TCEP is fixed to 1 mM and the temperature is varied, one can calculate reaction rate constants of the reduction process at each temperature. From the rates, one may calculate the rates of reduction of the disulfide bond of an individual $\beta_2m$ molecule by TCEP. This process – the encounter of the disulfide bond of $\beta_2m$ with TCEP molecules –
may also be simulated by computational molecular dynamics. Thus, experimental data and theory simulation results can be directly compared, which is rarely possible.

In order to measure reaction rates at a fixed temperature, I set up various β2m folding reactions. After ultracentrifugation, I diluted the soluble material in PBS and heated the solutions to different temperatures, namely 40 °C, 42.5 °C, 45 °C, and 50 °C. After equilibration for one hour, I added 1 mM TCEP to the solutions and recorded the tryptophan fluorescence intensity for at least 90 minutes. In all cases, I left a sample untreated at the respective temperature.

At 40 °C and below, there was almost no loss of fluorescence intensity after nearly two hours (Figure 5.5A, B; shown only until 80 minutes). At 42.5 °C, there was a minor decrease in fluorescence intensity. The loss in fluorescence intensity became evident at 45 °C, and stronger at 50 °C. The loss in fluorescence was very slow at all temperatures, reaching 85% after 80 minutes in the fastest reaction (50 °C). When I fitted a single exponential decay to each curve, without restricting the baseline as a boundary condition of the fit, the residual plots already showed some systematic deviations at 50 °C, although the normality test of the residuals indicated that the fit was correct (Table 5.1, condition 2). When I repeated the fitting procedure with various restrictions, such as the baseline to reach 30% of the initial signal (the baseline reached with acid, Figure 5.3), there were systematic deviations in all curves (Figure 5.5B), and the normality test of the residuals indicated that a mono-exponential model did not fit the data correctly (Table 5.1.) Thus, the reaction rates of reduction with 1 mM TCEP after heating at various temperatures are temperature dependent, but they cannot be fitted with accuracy under the experimental conditions evaluated.

5.2.5. The thermal denaturation and reduction of β2m with a low concentration of TCEP cannot be modelled as a single exponential decay at temperatures above 40°C

It is possible that in the previous experiment, the baseline was not reached because the measurements were not long enough. Thus, it was necessary to perform longer incubations. To do so, I performed a similar experiment to the one described in Figure 5.5A, but extending the measurement up to 540 minutes. Intriguingly, after 540 minutes of incubation at 40 °C in the presence of 1 mM TCEP, the baseline was not reached. Rather, the data seemed to be approaching a plateau (Figure 5.6A). When I fitted the data to a mono-exponential curve, there were systematic deviations in the residual plot, although the normality test indicated that
Figure 5.5 The accessibility of TCEP to the disulfide bond of β2m is temperature dependent.
After folding and ultracentrifugation, samples were heated up to the indicated temperatures and allowed to equilibrate for 60 minutes. Then 1 mM TCEP was added, and the intrinsic tryptophan fluorescence was recorded in the kinetic mode for at least 90 minutes. A. Fitting to a single exponential curve without baseline restrictions (Condition 2). Residual plots at all temperatures are shown below the fit. B. Fitting to a single exponential curve, where the baseline was constrained to reach 30% of the initial signal and the time point zero was constrained to 100% (Condition 5). Residual plots at all temperatures are shown below the fit. Dashes are the data, and black lines are the fit. Figure represents three or more independent experiments per curve, except 42.5 °C, that represent two independent experiments.
Table 5.1 Parameters of the mono-exponential fits at different temperatures.

The P-value was calculated using the built-in normality test D'Agostino-Pearson omnibus K2. The null hypothesis is that the residuals are normally distributed. If P ≥ 0.05, the residuals are normally distributed, and that indicates that the model fits the data.

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<td>4</td>
<td>Remove outliers, set up baseline to 30%</td>
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<tr>
<td>5</td>
<td>Remove outliers, set up baseline to 30%, and starting point to 100%</td>
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the fit was correct (Table 5.2). Thus, after 540 minutes, thermal denaturation and heating of β2m at 40 °C with 1 mM TCEP does not lead to a total loss of fluorescence, that is, to reach the baseline.

I next tested the hypothesis that if the concentration of TCEP present in the reaction is low enough to maintain some folded structure – as observed in Figure 5.4 – but higher than 1 mM, it might be possible to reach the spectroscopic baseline of denaturation faster. Thus, I performed a similar experiment, but in the presence of 3 mM TCEP, for up to 24 hours. Surprisingly, in the reaction to which I added 3 mM TCEP at 40 °C, there was another
process – at approximately 500 minutes – that was different from the initial exponential decay (Figure 5.6B). In the presence of 3 mM TCEP at 50 °C, the baseline was reached, in a process with faster kinetics that also clearly did not correspond to a single exponential decay. The denaturation of β2m upon treatment with 3 mM TCEP at 40 °C and 50 °C follows at least two different processes that cannot be modelled as a single exponential decay. Even in the absence of TCEP at 50 °C, the time courses of β2m yielded two different processes (Figure 5.6B). Taken together, my time course data indicate that the disulfide bond of β2m accessibility to TCEP (at a low concentration) at elevated temperatures is very low. Given that the denaturation and reduction of β2m cannot be modelled as a single exponential decay above 40 °C, it is not possible to describe the reactions occurring under these conditions with accuracy.

Since a simple model where the rate constants are clearly defined is not available, the conditions of experimentation have to be modified. Thus, in order to conclude the project, it is necessary to find adequate conditions where monomeric β2m can be evaluated, in such a way that no additional processes interfere with the actual measurement. Else, one can implement a model where further constraints are added. In summary, a substantial amount of work is necessary to continue with this line of investigation.

5.3. Discussion

Knowledge about the conformational flexibility of a protein is as important as the crystal structure. In order to evaluate the conformational flexibility of proteins, the methods available so far generally require large protein concentration, high purity, and complex machinery. These requirements are particularly important in protein NMR, which is the method that most accurately can measure protein mobility to the date.

Among the proteins used as model to study protein structure and dynamics, β2m is a popular example. Indeed, protein NMR studies have shown that β2m is particularly flexible when it is not bound to the heavy chain of MHC class I [15, 33]. In this work, the idea was to establish a simpler method that may in principle provide similar information about the dynamics of β2m in particular, and about proteins in general.

As a first step, avoiding aggregation problems is important. Protein aggregation is concentration-dependent, and if a critical concentration is achieved, aggregation may occur [4]. When the protein concentration in the refolding reaction was 500 µg/ml, and the
The thermal denaturation and reduction of hβ2m with low a concentration of TCEP cannot be modelled as single exponential decay.

After folding and ultracentrifugation, the soluble material was heated up to 40 °C or 50 °C, respectively. Then, the samples were allowed to equilibrate for 60 minutes at the respective temperature, and A. 1 mM TCEP or B. 3 mM TCEP were added or not. Finally, the intrinsic tryptophan fluorescence was recorded in the kinetic mode for A. 550 minutes B. 1500 minutes. In A, the curve was fitted to a single exponential decay, and the baseline was not constrained. Residual plot is below the curve. Dashes are the data, and black lines are the fit. Figure represents two independent curves at 40 °C, and n=1 at 50 °C.

Figure 5.6 The thermal denaturation and reduction of hβ2m with low concentration of TCEP cannot be modelled as single exponential decay.
Table 5.2 Parameters of the mono exponential fit, 40 °C, 1 mM TCEP, 550 minutes, condition 2

<table>
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<th>Parameter</th>
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<td>Starting point (%)</td>
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<td>Baseline (%)</td>
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protein solution was dialyzed against PBS pH 7.4, disulfide-bonded oligomers were formed (Figure 5.2A).

This is consistent with previous observations [31], but is not desirable for the analysis of conformational flexibility of monomeric β₂m. When the concentration was reduced, and the dialysis step was omitted, the monomeric form of β₂m was the only visible species observed in the gel stained with Coomassie Blue (Figure 5.2B). It is possible that both effects individually, namely, the decrease in the concentration and the lack of dialysis had contributed to the exclusive formation of β₂m monomers.

At room temperature, when β₂m molecules present in the solution are treated with 10 mM TCEP, the fluorescence intensity decreases to 50% (Figure 5.3). This may mean that either all β₂m molecules in the solution are reduced but not totally unfolded, or else that some molecules are completely unfolded after reduction, and the remaining folded molecules yield the remaining fluorescence. Since in the reaction there is thousand-fold molar excess of TCEP (10 mM TCEP vs. approximately 7 μM β₂m), it is more likely that the vast majority of the β₂m molecules in solution have been reduced, but that they retain some partial structure that has not unfolded in the time frame of the experiment, and that gives the residual fluorescence.

Importantly, the reduction of the disulfide bond of β₂m at room temperature is a slow process [28, 29]. Even at 37 °C, in an experiment done by Gozu et al. [29], it took nearly 420 minutes to detect the completely reduced form of 20 μM β₂m after treatment with 180 mM DTT at pH 8.5. This might also explain why there is no total denaturation observed after reduction of β₂m.
All these data, taken together, imply that the disulfide bond in β₂m is protected from reduction at temperatures below 37 °C. This indicates that below 37 °C, the conformational flexibility of β₂m is relatively low.

In the presence of a low concentration of TCEP (1 mM), β₂m is less thermally stable (by 8 °C) than the untreated protein (Figure 5.4). This result suggests that when the temperature increases, the accessibility of the disulfide bond to TCEP molecules increases. This is the most important observation of this work, because it means that the global flexibility of a protein can be examined in vitro without complex methods such as protein NMR. (An alternative explanation to increased conformational flexibility upon temperature increase is that β₂m very slowly changes its conformation into another (rigid) state in which the disulfide bond is accessible to TCEP (and which then denatures). This experiment does not provide a way to differentiate whether this is occurring. Thus, this alternative cannot be excluded. To prove this hypothesis, then protein NMR is required.)

When a higher concentration of TCEP (10 mM) was added in an analogous TDTF experiment, no denaturation signal was detected upon raising the temperature. The simplest explanation of this result is that when β₂m was treated with 10 mM TCEP, the denaturation and unfolding occurred already at low temperature, before the experiment or at its very beginning (below 30 °C). In agreement with this hypothesis, an experiment where 20 µM β₂m was pre-treated with 4 M guanidinium hydrochloride and then reduced with 0.5 mM DTT at room temperature yielded a completely unfolded protein after only 10 minutes [29].

In a single experiment where I increased the heating rate to 1 °C/min, and then recorded a TDTF curve in the presence of 1 mM TCEP, I did not observe changes in the Tₘ compared to 0.33 °C/min (data not shown). Thus, even though denaturation appeared slow in some of the curves of Figure 5.6 (taken at 40 °C or 50 °C), it was at 54 °C still fast in the time frame of the TDTF experiment. This experiment has to be repeated, as well as an experiment in which the heating rate is decreased.

Importantly, the reduction process at temperatures greater than 40 °C cannot be modeled as a single exponential decay when the baseline is fixed to 30% of the initial signal (Figure 5.5B, Figure 5.6A, residual plots, and Table 5.1). This hindered further development of this approach, since no accurate rate constants can be calculated from the data. The fact that the
curves cannot be modeled as a single exponential decay implies that there is more than one process happening at the same time.

When the concentration of TCEP was increased to 3 mM, the T_m did not change (not shown). In the time course experiments shown on Figure 5.6 there are at least two visible processes. A possible interpretation of these results is that there are two processes, namely, one slow and another one fast. The slow process might be the - slow – reduction of the disulfide bond that is also visible in Figure 5.5 and Figure 5.6A, and the fast process might be the aggregation of the reduced molecules in the solution. These reduced molecules might accumulate and serve as the template of a structural nucleus [46] to form non-covalent oligomers. This data is consistent with the idea that upon fluctuations (unfolding) in the protein structure, aggregation-prone sequences become exposed to the solvent [12]. Even the agitation of a protein for long time at room temperature can promote aggregation [47].

Evidently, there is much more work to be done in order to understand what is happening in this reaction, and how can this be interpreted in terms of the flexibility of β_2m. To continue this project, several experimental parameters have to be varied. The first parameter that needs to be controlled is the initial purification of the protein. While the preparation of β_2m by the protocol described in [27] has proven valuable to identify conformational properties of MHC class I [48], it is possible that even small impurities present in the refolding reaction trigger the formation of aggregates at high temperatures. Thus, a gel filtration protocol to purify β_2m is desirable as a next step.

Once the protein is purified, the same experiments performed here can be repeated, and the likelihood to obtain reaction rate constants of reduction after thermal denaturation will be higher. In order to identify the presence of reduced and oxidized species of β_2m by an independent method, it will be ideal to establish a RP-HPLC system such as the one reported in [29]. If the fast processes shown in Figure 5.6B are indeed aggregation processes, then it should be possible to delay or suppress them by further decreasing the protein concentration.

At this point, molecular dynamics simulations might be useful such as those described previously, for example in the characterization of β_2m homodimers [32]. Ideally, one should start by the analysis of the RMSD trajectories of monomeric β_2m while the temperature is varied in intervals of 50 K up to the maximal temperature admissible by the program, in a similar way as earlier reports [49]. This knowledge will be useful to identify the effect of heat
on the conformational flexibility of β\textsubscript{2}m. Once this is known, simulations with TCEP can be performed. Initially, TCEP molecules could be added into the water box with β\textsubscript{2}m. In the Springer group, molecular dynamics simulations of β\textsubscript{2}m have been done in a water box that contains 5,443 water molecules (Esam Tolba Abualrous, personal communication). In such a water box, a single molecule of TCEP will be at a concentration of approximately 10 mM. The parameter to follow will be the distance between the disulfide bond of β\textsubscript{2}m and individual TCEP molecules over time. At 300 K (26.8 °C), it is very likely that TCEP molecules bind to the surface of β\textsubscript{2}m, but that they have no access to the disulfide bond. Once the temperature is increased and the conformation of β\textsubscript{2}m fluctuates, we will perhaps observe collisions of the TCEP molecule(s) with the disulfide bond in the center of the protein. At higher temperatures, there would presumably be more collisions, and thus one could try to correlate the difference in the collision rates with the difference in the denaturation rates derived from the experiment. A simple linear relationship would look like this:

\[
\frac{C_{50}}{C_{40}} = p \times \frac{k_{50}}{k_{40}}
\]

with C\textsubscript{x} = collision rate in simulation at x °C; k\textsubscript{x} = denaturation rate constant at x °C, and p the proportionality constant.

Importantly, the correlation between experiments and simulations can be a powerful tool to study the origin and the solution of amyloids at the molecular level, not only in the context of β\textsubscript{2}m, but also in many other amyloidogenic proteins.

### 5.4. Materials and Methods

**TCEP.** TCEP was from Applichem (Darmstadt, Germany). Stock solutions of TCEP were prepared freshly for each experiment in 50 mM Tris·Cl pH 8.0, as recommended by the manufacturer.

**β\textsubscript{2}m expression, purification, and refolding.** The full protocol is described in [27]. Briefly, human β\textsubscript{2}m was produced in E.coli BL21(DE3) under the control of the T7 promoter. Cells were spun down and sonicated in sucrose buffer (25% sucrose, 1 mM EDTA, 1 mM PMSF, and 10 mM DTT in 10 mM Tris·Cl, pH 8.0). Inclusion bodies were harvested by centrifugation at 40,000 x g for 15 minutes, washed in detergent buffer (25% sucrose, 1% Triton X 100, 5 mM EDTA, and 2 mM DTT in 50 mM Tris·Cl pH 8.0), urea buffer (2 M
NaCl, 2 M urea, and 2 mM DTT, in 25 mM Tris·Cl pH 8.4), TBS (150 mM NaCl and 0.5 mM PMSF in 20 mM Tris·Cl pH 7.5), and then dissolved in 8 M urea (with 50 mM K-HEPES pH 6.5 and 100 µM β-mercaptoethanol). After centrifugation at 40 000 x g for 15 minutes, the soluble denatured protein was stored at -80 °C until use. The refolding reactions were performed in 2 ml of refolding buffer (100 mM Tris·Cl pH 8, 0.5 M arginine, 2 mM EDTA, 0.5 mM oxidized glutathione, 5 mM reduced glutathione). The reaction was incubated at 4 °C for at least 48 hours, then ultracentrifuged at 100 000 x g for 20 minutes to sediment aggregates. Typically, after ultracentrifugation, there was no visible pellet. The concentration used for each refolding reaction is stated in every figure legend. In the dialysis experiment, dialysis was done for two days against 1x PBS, with four changes of buffer in between.

**Determination of Spectra.** After ultracentrifugation, refolding reactions in refolding buffer were diluted with three parts of PBS, for a final volume of 2 ml. The solutions were placed in a 1 cm x 1 cm cuvette with a stir bar, and TCEP or HCl were added to the solutions and mixed by pipetting. Immediately afterwards, the cuvettes were placed in a Cary Eclipse fluorimeter (Agilent, Waldbronn, Germany), and emission spectra were recorded at an excitation of 290 nm, in a range from 290 to 600 nm. The data shown in Figure 5.3 does not include the Raman peak present at 550 nm.

**Thermal Denaturation measured by Tryptophan Fluorescence.** After ultracentrifugation, refolding reactions in refolding buffer were diluted with three parts of PBS, for a final volume of 2 ml. Then, the kinetic mode was activated and the intrinsic tryptophan fluorescence intensity was recorded at room temperature for a minute, with an excitation of 290 nm and emission of 345 nm. 50 µl of the respective TCEP stock solution were added to the samples in the cuvettes, and the fluorescence intensity was recorded for another minute. Finally, the thermal mode was activated, and TDTF curves were recorded as in [27].

**Determination of TCEP kinetics at fixed temperatures.** Samples were diluted as above, and examined for a minute in the kinetic mode before the thermal mode was activated. Next, the protein solutions were heated up to the desired temperature at a heating rate of 0.3 °C/min. When the temperature was reached, the kinetic mode was activated again, and the fluorescence intensity was recorded for an hour at the respective temperature. After exactly 60 minutes, 50 µL of the respective stock solution of TCEP were added accordingly, and the fluorescence intensity was recorded for the described time periods.
SDS-PAGE. After ultracentrifugation (and dialysis where indicated), an aliquot of the soluble material was taken. The non-reducing buffer was 4x Laemmli sample buffer, 20% SDS, no DTT. The reducing buffer was identical, but with 20 mM DTT. The respective buffers were added to the samples; then, the samples were heated up 5 minutes at 95 °C, cooled down to room temperature, and 50 mM N-ethylmaleimide was added to all samples to prevent re-oxidation, for 10 minutes. Empty lanes in the gel were loaded with non-reducing buffer. A 16 cm long SDS-PAGE of 3 steps was prepared, from bottom to top: 15%, 10%, and 5%. Gels were run overnight at 10 mA. Staining of the gel was with Coomassie Blue.

Data analysis. TDTF curves were processed as published in [27], using a LOWESS fit. For the kinetic experiments, the initial fluorescence intensity of individual experiments was normalized to 100% and averaged across experiments. The resulting curves were fitted to a single exponential decay, either fixing the baseline to 30% of the initial fluorescence or leaving the algorithm to fit it. The initial value of the fitting was also fixed to 100% or left to the algorithm to fit. Outliers were removed by the algorithm when indicated. Residuals plots are the result of the model minus the averaged fluorescence intensity at each time point. The normality test was the built-in D'Agostino-Pearson omnibus K2, as recommended by the manufacturer. All calculations were done with GraphPad Prism 6 (GraphPad Software, Inc., San Diego, USA). Figures were drawn with Inkscape (http://www.inkscape.org).
5.5. References


23. Raimondi, S., et al., The two tryptophans of beta2-microglobulin have distinct roles in function and folding and might represent two independent responses to evolutionary pressure. BMC Evol Biol, 2011. 11: p. 159.


