Peptide-dependent trafficking of MHC class I molecules

by

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of the requirements for the degree of
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The distribution of endogenous class I molecules (green) in wild type (T1) lymphocytes at 20°C in respect to the ER exit sites (red).

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Abstract

Major histocompatibility complex (MHC) class I molecules are central to the immune system. They present endogenous antigen in the form of peptides to cytotoxic T lymphocytes (CTL).

Peptides bind to class I molecules with different affinities that depend on their length and sequence, with low-affinity peptides having higher dissociation rates. After synthesis, class I molecules first appear to bind to low-affinity peptides and then to exchange them for higher-affinity ones. Low-affinity peptides might dissociate from class I molecules during the transport to the cell surface, and peptide-receptive class I molecules at the cell surface might bind to extracellular peptides. Therefore, cells keep peptide-receptive forms of class I in their interior by an unknown mechanism, either by retention in the ER, or by retrieval from the ERGIC or the cis-Golgi. This control mechanism involves the so-called class I loading complex, which consists of four proteins: the peptide transporter TAP, and the chaperones calreticulin, ERp57, and tapasin. To elucidate where and how empty class I is retained in the cell, I have used confocal fluorescence microscopy.

I have found that all forms of class I can leave the endoplasmic reticulum and become transported to the cis-Golgi, from where only those forms that are bound to high-affinity peptides can proceed to the cell surface, while peptide-receptive forms are retrieved to the ER. This finding is valid for both endogenous and overexpressed class I molecules. An interesting new finding is that in hamster (CHO) and monkey (Vero) fibroblasts, transfected murine class I molecules are mostly localized intracellularly. This is probably because the transfected mouse class I molecules cannot interact efficiently with the endogenous hamster loading complex and, as a consequence, cannot efficiently exchange low-affinity peptides for higher-affinity ones; thus, they cannot be exported to the cell surface. With the help of Irina Majoul at Royal Holloway University, I have developed a method to introduce high-affinity peptides into these cells by electroporation. This treatment moves mouse class I to the cell
surface in hamster and monkey fibroblasts. I have then checked different peptides in their ability to move class I to the cell surface, and I have found that the carboxy terminus of the peptide is important for the export of the class I to the cell surface.

I have also investigated the effect of tapasin and calreticulin, components of the peptide loading complex, on the trafficking of class I. In both tapasin–negative and calreticulin–negative cell lines, class I remains intracellular. Interestingly, introduction of high-affinity peptides can partially overcome this defect in both cell lines. That confirms the role of tapasin and calreticulin in the exchange of the peptides bound to class I from low-affinity to higher-affinity peptides.
Acknowledgments

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# Table of contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Introduction</td>
<td>1</td>
</tr>
<tr>
<td>1.1. The role of class I molecules</td>
<td>1</td>
</tr>
<tr>
<td>1.2. The structure of MHC class I molecules</td>
<td>1</td>
</tr>
<tr>
<td>1.3. Assembly of MHC class I molecules</td>
<td>3</td>
</tr>
<tr>
<td>1.4. Trafficking of MHC class I molecules</td>
<td>4</td>
</tr>
<tr>
<td>1.5. The generation of peptides and their binding to class I molecules</td>
<td>9</td>
</tr>
<tr>
<td>1.5.1. Peptide generation</td>
<td>9</td>
</tr>
<tr>
<td>1.5.2. Peptide delivery to the ER</td>
<td>10</td>
</tr>
<tr>
<td>1.5.2.1. Peptide size</td>
<td>10</td>
</tr>
<tr>
<td>1.5.2.2. Peptide sequence</td>
<td>11</td>
</tr>
<tr>
<td>1.5.3. Peptide binding to class I</td>
<td>11</td>
</tr>
<tr>
<td>1.5.3.1. Peptide size</td>
<td>11</td>
</tr>
<tr>
<td>1.5.3.2. Peptide sequence</td>
<td>12</td>
</tr>
<tr>
<td>1.5.3.2.1. Anchor residues</td>
<td>12</td>
</tr>
<tr>
<td>1.5.3.2.2. N- and C-termini</td>
<td>13</td>
</tr>
<tr>
<td>1.6. The role of the chaperone molecules in peptide optimization and trafficking of peptide-receptive class I molecules</td>
<td>14</td>
</tr>
<tr>
<td>1.6.1. Calnexin</td>
<td>14</td>
</tr>
<tr>
<td>1.6.2. Calreticulin</td>
<td>16</td>
</tr>
<tr>
<td>1.6.3. ERP57</td>
<td>17</td>
</tr>
<tr>
<td>1.6.4. The transporter associated with antigen processing (TAP)</td>
<td>20</td>
</tr>
<tr>
<td>1.6.5. Tapasin</td>
<td>21</td>
</tr>
<tr>
<td>1.7. The project of the thesis</td>
<td>24</td>
</tr>
<tr>
<td>2. Materials and methods</td>
<td>25</td>
</tr>
<tr>
<td>2.1. Materials</td>
<td>25</td>
</tr>
<tr>
<td>2.1.1. Cell lines</td>
<td>25</td>
</tr>
<tr>
<td>2.1.2. Fluorescent constructs</td>
<td>26</td>
</tr>
<tr>
<td>2.1.3. Antibodies</td>
<td>27</td>
</tr>
<tr>
<td>2.1.4. Pure peptides</td>
<td>28</td>
</tr>
</tbody>
</table>
### 2.2. Methods

<table>
<thead>
<tr>
<th>Subsection</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.2.1. Generation of expression plasmids</td>
<td>29</td>
</tr>
<tr>
<td>2.2.1.1. Amplification of H-2K\textsuperscript{b} and H-2D\textsuperscript{b} cDNA</td>
<td>29</td>
</tr>
<tr>
<td>2.2.1.2. Restriction digest and ligation of H-2K\textsuperscript{b} and H-2D\textsuperscript{b} inserts and pEGFP-N1 and Ds-Red-N1 vectors</td>
<td>30</td>
</tr>
<tr>
<td>2.2.1.3. Preparation of chemically competent E.Coli cells for transformation</td>
<td>31</td>
</tr>
<tr>
<td>2.2.1.4. Transformation of competent cells</td>
<td>31</td>
</tr>
<tr>
<td>2.2.1.5. Diagnostic digest</td>
<td>32</td>
</tr>
<tr>
<td>2.2.1.6. Maxi-preparation of plasmids</td>
<td>33</td>
</tr>
<tr>
<td>2.2.2. Transfections</td>
<td>35</td>
</tr>
<tr>
<td>2.2.3. Immunofluorescence microscopy</td>
<td>36</td>
</tr>
<tr>
<td>2.2.3.1. Colocalization experiments</td>
<td>36</td>
</tr>
<tr>
<td>2.2.3.1.1. Preparation of cells</td>
<td>36</td>
</tr>
<tr>
<td>2.2.3.1.1.1. Preparation of suspension cells</td>
<td>36</td>
</tr>
<tr>
<td>2.2.3.1.1.2. Preparation of adherent cells</td>
<td>37</td>
</tr>
<tr>
<td>2.2.3.1.2. Blocking protein transport in organelles</td>
<td>38</td>
</tr>
<tr>
<td>2.2.3.2. Electroporation of the peptide</td>
<td>38</td>
</tr>
<tr>
<td>2.2.3.3. Microscopic observations settings</td>
<td>39</td>
</tr>
</tbody>
</table>

### 3. Results

<table>
<thead>
<tr>
<th>Subsection</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1. Trafficking of MHC class I molecules</td>
<td>41</td>
</tr>
<tr>
<td>3.1.1. Trafficking of MHC class I molecules in lymphocytes</td>
<td>42</td>
</tr>
<tr>
<td>3.1.2. Trafficking of MHC class I molecules in fibroblasts</td>
<td>46</td>
</tr>
<tr>
<td>3.2. Peptide effect on the class I localization</td>
<td>49</td>
</tr>
<tr>
<td>3.2.1. Peptide can access the ER only upon electroporation</td>
<td>50</td>
</tr>
<tr>
<td>3.2.2. Peptide is electroporated to the cytosol and then transported to the ER by TAP transporter</td>
<td>51</td>
</tr>
<tr>
<td>3.2.3. Peptide relocates class I from the ER to the Golgi apparatus and the cell surface</td>
<td>52</td>
</tr>
<tr>
<td>3.2.4. The carboxy terminus of the peptide is required for the export of peptide-class I complex to the cell surface</td>
<td>52</td>
</tr>
<tr>
<td>3.3. The effect of tapasin on class I localization</td>
<td>56</td>
</tr>
<tr>
<td>3.4. The effect of calreticulin on class I localization</td>
<td>58</td>
</tr>
<tr>
<td>-----------------------------------------------------</td>
<td>----</td>
</tr>
<tr>
<td>3.5. Results figures</td>
<td>60</td>
</tr>
<tr>
<td>4. Discussion</td>
<td>79</td>
</tr>
<tr>
<td>4.1. Trafficking of MHC class I molecules</td>
<td>79</td>
</tr>
<tr>
<td>4.2. The peptide effect on class I export from the ER, and the class I ER export signal</td>
<td>88</td>
</tr>
<tr>
<td>4.3. The features of peptide that determine exit of the peptide-class I complex to the cell surface</td>
<td>89</td>
</tr>
<tr>
<td>4.4. The effect of tapasin on class I export to the cell surface</td>
<td>92</td>
</tr>
<tr>
<td>4.5. The effect of calreticulin on class I export to the cell surface</td>
<td>96</td>
</tr>
<tr>
<td>4.6. The role of the components of the loading complex in peptide optimization and class I trafficking</td>
<td>98</td>
</tr>
<tr>
<td>5. Further research</td>
<td>102</td>
</tr>
<tr>
<td>6. References</td>
<td>104</td>
</tr>
</tbody>
</table>
## List of figures

<table>
<thead>
<tr>
<th>Figure</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1</td>
<td>The structure of MHC class I molecules H-2D(^b) with FAPGYPAL peptide</td>
<td>2</td>
</tr>
<tr>
<td>Figure 2</td>
<td>Biogenesis of trimeric class I molecules, and the retention model of MHC class I trafficking</td>
<td>5</td>
</tr>
<tr>
<td>Figure 3</td>
<td>Retrieval model of MHC class I trafficking</td>
<td>7</td>
</tr>
<tr>
<td>Figure 4</td>
<td>The distribution of endogenous class I molecules in wild type and TAP-deficient lymphocytes at 37 °C</td>
<td>60</td>
</tr>
<tr>
<td>Figure 5</td>
<td>The distribution of endogenous class I molecules in wild type and TAP-deficient lymphocytes at 15 °C</td>
<td>61</td>
</tr>
<tr>
<td>Figure 6</td>
<td>The distribution of endogenous class I molecules in wild type and TAP-deficient lymphocytes at 20 °C</td>
<td>62</td>
</tr>
<tr>
<td>Figure 7</td>
<td>The distribution of H-2D(^b) molecules in wild type and TAP-deficient fibroblasts at 37 °C</td>
<td>63</td>
</tr>
<tr>
<td>Figure 8</td>
<td>The distribution of H-2D(^b) molecules in wild type and TAP-deficient fibroblasts at 15 °C</td>
<td>64</td>
</tr>
<tr>
<td>Figure 9</td>
<td>The distribution of H-2D(^b) molecules in wild type and TAP-deficient fibroblasts at 20 °C</td>
<td>65</td>
</tr>
<tr>
<td>Figure 10</td>
<td>H-2K(^b)-GFP relocalizes from the ER to the cell surface of fibroblasts following electroporation with SIINFEKL peptide</td>
<td>66</td>
</tr>
<tr>
<td>Figure 11</td>
<td>The peptide delivery to the ER is TAP dependent</td>
<td>67</td>
</tr>
<tr>
<td>Figure 12</td>
<td>Time course of the H-2D(^b)-FAPGYPAL transport to the cell surface</td>
<td>69</td>
</tr>
<tr>
<td>Figure 13</td>
<td>Not all peptides induce the transport of class I to the cell surface</td>
<td>70</td>
</tr>
<tr>
<td>Figure 14</td>
<td>FAPGYP can not move class I to the cell surface, but it competes with NYPAL for binding to class I</td>
<td>72</td>
</tr>
<tr>
<td>Figure 15</td>
<td>The tapasin effect on the distribution of class I</td>
<td>74</td>
</tr>
<tr>
<td>Figure 16</td>
<td>FAPGYP competes with endogenous peptides for binding to class I</td>
<td>76</td>
</tr>
<tr>
<td>Figure 17</td>
<td>The calreticulin effect on the distribution of class I</td>
<td>77</td>
</tr>
<tr>
<td>Figure 18</td>
<td>Trafficking of MHC class I molecules</td>
<td>81</td>
</tr>
<tr>
<td>Figure 19</td>
<td>The structure of the peptide binding groove of H-2D(^b) with the FAPGYPAL peptide and predicted structure of the binding groove without peptide, with NYPAL, or with FAPGYP peptide</td>
<td>91</td>
</tr>
</tbody>
</table>
List of tables

<table>
<thead>
<tr>
<th>Table</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 1</td>
<td>Fluorescent constructs used in colocalization experiments</td>
<td>26</td>
</tr>
<tr>
<td>Table 2</td>
<td>Antibodies against organelle markers</td>
<td>27</td>
</tr>
<tr>
<td>Table 3</td>
<td>Antibodies against class I</td>
<td>27</td>
</tr>
<tr>
<td>Table 4</td>
<td>Secondary antibodies</td>
<td>27</td>
</tr>
</tbody>
</table>

Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABC</td>
<td>ATP binding cassette transporter</td>
</tr>
<tr>
<td>APC</td>
<td>antigen presenting cell</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>B₂m</td>
<td>beta-2 microglobulin</td>
</tr>
<tr>
<td>B₂m-NF</td>
<td>beta-2-negative fibroblasts</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumine</td>
</tr>
<tr>
<td>°C</td>
<td>degrees Celcius</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese Hamster Ovary (cells)</td>
</tr>
<tr>
<td>COP</td>
<td>COP protein complex</td>
</tr>
<tr>
<td>CTL</td>
<td>cytotoxic T lymphocyte</td>
</tr>
<tr>
<td>CNX</td>
<td>calnexin</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>Endo H</td>
<td>Endoglycosidase H</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>ERAP</td>
<td>ER associated peptidase</td>
</tr>
<tr>
<td>ERGIC</td>
<td>ER-Golgi intermediate compartment</td>
</tr>
<tr>
<td>FCS</td>
<td>fetal calf serum</td>
</tr>
<tr>
<td>GalT</td>
<td>Galactosyl-transferase</td>
</tr>
<tr>
<td>G418</td>
<td>gentamicin</td>
</tr>
<tr>
<td>h</td>
<td>hours</td>
</tr>
<tr>
<td>HC</td>
<td>heavy chain</td>
</tr>
<tr>
<td>Symbol</td>
<td>Definition</td>
</tr>
<tr>
<td>--------</td>
<td>------------</td>
</tr>
<tr>
<td>$K_D$</td>
<td>equilibrium dissociation constant, $k_{off}/k_{on}$</td>
</tr>
<tr>
<td>kDa</td>
<td>kilo Daltons</td>
</tr>
<tr>
<td>K41</td>
<td>wild type mouse fibroblasts, parental of K41</td>
</tr>
<tr>
<td>K42</td>
<td>calreticulin-negative mouse fibroblasts, derivative of K41</td>
</tr>
<tr>
<td>M</td>
<td>molar</td>
</tr>
<tr>
<td>MEF</td>
<td>mouse ear fibroblasts</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>min</td>
<td>minutes</td>
</tr>
<tr>
<td>n</td>
<td>nano</td>
</tr>
<tr>
<td>NBD</td>
<td>nucleotide binding domain</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PDI</td>
<td>protein disulfide isomerase</td>
</tr>
<tr>
<td>PLC</td>
<td>peptide loading complex</td>
</tr>
<tr>
<td>PSG</td>
<td>penicillin/streptomycin/glutamine</td>
</tr>
<tr>
<td>RMA</td>
<td>mouse lymphoid cell line, parental of RMA-S</td>
</tr>
<tr>
<td>RMA-S</td>
<td>TAP2-deficient mouse lymphoid cell line, derivative of RMA</td>
</tr>
<tr>
<td>T1</td>
<td>human lymphoid cell line, parental of T2</td>
</tr>
<tr>
<td>T2</td>
<td>TAP2-deficient human lymphoid cell line, derivative of T1</td>
</tr>
<tr>
<td>TAP</td>
<td>transporter associated with antigen processing</td>
</tr>
<tr>
<td>TAP2d</td>
<td>TAP2-deficient (cells)</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris Buffered saline</td>
</tr>
<tr>
<td>$T_M$</td>
<td>melting temperature</td>
</tr>
<tr>
<td>TMD</td>
<td>transmembrane domain</td>
</tr>
<tr>
<td>TAPBP-R</td>
<td>TAP binding protein related</td>
</tr>
<tr>
<td>Tapasin</td>
<td>TAP associated protein</td>
</tr>
<tr>
<td>TPN, tpn</td>
<td>Tapasin</td>
</tr>
<tr>
<td>TPN&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>Tapasin negative</td>
</tr>
<tr>
<td>Vero</td>
<td>African green monkey kidney (cells)</td>
</tr>
</tbody>
</table>

Abbreviations for amino acids and chemical elements follow standard nomenclature.
1. **Introduction**

1.1. **The role of class I molecules**

Major histocompatibility complex (MHC) class I molecules play a central role in the mammalian immune system. They allow the immune system to monitor the presence of pathogens – viruses, bacteria and other parasites – that multiply in the host-cell cytoplasm. The process starts in the proteasome where all proteins are continuously degraded into small peptides. These peptides are transported into the lumen of the endoplasmic reticulum (ER) by the transporter associated with antigen presentation (TAP). In the ER, peptides are bound to MHC class I molecules, and the resulting complex is transported to the cell surface and presented to cytotoxic T lymphocytes (CTL). If a peptide is derived from a nonself protein (or, in autoimmune diseases, from a self protein to which no tolerance exists), it will be recognized by a T cell receptor on a CTL which will then induce the apoptosis of the infected cell (Pamer and Cresswell, 1998) and thus prevent the pathogen from spreading. Ten to a hundred class I-peptide complexes at the cells surface are enough to induce a CTL response (Yewdell et al., 2003).

1.2. **The structure of MHC class I molecules**

MHC class I molecules are heterodimers that are composed of a heavy chain (Figure 1, in red), a light chain (beta-2 microglobulin, β₂m, Figure 1, in pink), and an antigenic peptide of eight to ten amino acids (Figure 1, in blue). The heavy chain is a polymorphic transmembrane protein. Many different alleles exist
at the three gene loci (HLA-A, B, C in human, and H-2D, K, and L in mice), whereas \( \beta_2 \text{m} \) is invariant.

The membrane–anchored heavy chain consists of three extracellular domains that are named \( \alpha_1 \), \( \alpha_2 \), and \( \alpha_3 \). The \( \alpha_3 \) domain and \( \beta_2 \text{m} \) together support the \( \alpha_1 \) and \( \alpha_2 \) domains, which form an antiparallel \( \beta \)-sheet with two helices on top between which there is the peptide binding groove. The antigenic peptide binds in this groove and interacts with MHC class I residues along the binding site through its main- and side-chain atoms (Bouvier and Wiley, 1994; Guo et al., 1993; Parker et al., 1992; Ruppert et al., 1993; and Saito et al., 1993).

Figure 1. The structure of an MHC class I molecule H-2D\(^{b}\) with the FAPGNYPAL peptide (sequence taken from Protein Data Bank, number 1CE6). The picture was done by Florian Sieker.

Two features of class I molecules are crucial for its proper assembly: glycosylation sites and disulfide bonds. All class I molecules are glycosylated at asparagine 86 (some murine alleles also at residues 176 and/or 256). The region of the \( \alpha_1 \) domain that contains the Asn-linked glycosylation site is recognized by the ER lectins calnexin and calreticulin, which are required for the proper folding of MHC class I molecules. Mutations of residues 86-88 prevent class I glycosylation and abolish its association with calreticulin (Harris et al., 1998; Harris et al., 2001).
The MHC class I heavy chain contains four conserved cysteine residues: Cys101 and Cys164 within the $\alpha_2$ domain and Cys203 and Cys259 in the $\alpha_3$ domain. These cysteine residues are recognized by the protein disulfide isomerase ERp57, which mediates disulfide bond formation within class I heavy chain (Hughes and Cresswell, 1998; Lindquist et al., 1998; Morrice and Powis, 1998).

1.3. Assembly of MHC class I molecules

Figure 2 gives an overview of the assembly and trafficking of class I molecules. The heavy chain is cotranslationally inserted into the membrane of the endoplasmic reticulum, with the $\alpha_1$-$\alpha_3$ domains in the lumen. It is assisted by BiP, which helps its proper folding and prevents premature degradation (Matlack et al., 1998). Like with most glycoproteins, the sugar chain is then processed to the monoglucosylated form by the ER-resident enzymes, glucosidase I and glucosidase II. The trimming of the last glucose residue serves as the recognition signal for the binding of calnexin and (in complex with it) the protein disulfide isomerase, ERp57 (Tector and Salter, 1995). Calnexin was shown to bind to the free heavy chain (Leach et al., 2002; Leach and Williams, 2004) and to recruit ERp57, which facilitates the formation of disulfide bonds in the heavy chain (Farmery et al., 2000). Next, $\beta_2m$ binds to the heavy chain (it contacts both the $\alpha_3$ and the $\alpha_1/\alpha_2$ domains), and the dimer dissociates from calnexin to associate with calreticulin. It is unknown whether at this stage, calreticulin-bound class I is truly empty or already occupied with low-affinity peptides. To bind optimal peptides, class I HC-$\beta_2m$ complexes associate (in addition to calreticulin and ERp57) also with TAP and tapasin (the TAP-associated protein) within the so-called peptide
loading complex (Diedrich et al., 2001). Tapasin is crucial for the binding of high-affinity peptides. In cells that lack tapasin, most class I molecules, for example HLA-B*4402, cannot bind high-affinity peptides, and they are not expressed on the cell surface. They do not reach the medial Golgi since they remain sensitive to endoglycosidase H (EndoH) digestion. Tapasin facilitates optimal peptide binding to the MHC class I/β2m dimer, thus allowing peptide-bound forms to be transported to the cell surface (reviewed by Cresswell et al., 1998; and Wright et al., 2004).

It is reasonable to assume that the molecular mechanism of the export of class I molecules from the ER is similar to that of other membrane glycoproteins (Garstka et al., 2007). To leave the ER, they are packaged into COPII (coat protein complex II) transport vesicles. COPII vesicles travel to the ER-Golgi intermediate compartment (ERGIC), and from there, the peptide-bound forms are transported to the Golgi apparatus and then to the cell surface where they act in antigen presentation to cytotoxic T cells.

1.4. Trafficking of MHC class I molecules

MHC class I molecules bound with high affinity peptides are transported to the cell surface, where they present antigen to CTL. To avoid being killed by a foreign peptide taken from the environment and to ensure that class I molecules are used efficiently, cells do not normally allow peptide-free forms to proceed to the cell surface, and only a small fraction of peptide-receptive class I molecules can be found at the cell surface (Yu et al., 1999). In many cases, these empty
class I molecules at the cell surface seem to be formed from class I-peptide complexes from which the peptide has dissociated at the cell surface.

Figure 2: Biogenesis of trimeric class I molecules, and the retention model of MHC class I trafficking. In human cells, class I heavy chains first associate with calnexin (CNX) and ERp57 (1) and then bind β2m (2). The resulting dimers bind calreticulin (CRT), the TAP peptide transporter, and tapasin (4). In this "loading complex", they bind to peptides generated by the proteasome in the cytosol. In the retention model, only peptide-bound class I molecules can enter COPII vesicles for transport to the Golgi apparatus, but empty dimers cannot (Figure courtesy of Patrycja Kozik and Sebastian Springer).

In the ER, there are three different forms of MHC class I molecules (free heavy chain, HC-β2m dimer, and the fully assembled complex of class I and peptide), but only the fully assembled complex undergoes the sugar modifications by mannosidase II in the medial Golgi which render it endoglycosidase H resistant and proceeds further to the cell surface, while free heavy chains and empty dimers appear to have no access to the medial-Golgi and the later stages of the secretory pathway, since they do not acquire EndoH resistance (Baas et al.,
1992). This suggests that the peptide is critical for the class I export to the cell surface. But is it necessary for the export of MHC class I from the endoplasmic reticulum?

To approach this question, one needs to determine whether peptide-free forms can leave the ER and, therefore, one needs to investigate the ER export of free heavy chains and HC-β2m dimers. Free heavy chains interact with calnexin and ERp57 for their proper folding, and because of the action of these chaperone molecules, class I heavy chains can bind β2-microglobulin. Since the binding of β2m and the peptide is cooperative, one would assume that heavy chains do not bind peptides to the same extent as HC-β2m dimers do. When β2m is bound to the heavy chain, the dimer can bind peptide without the help of the components of the peptide loading complex (PLC), at least in vitro (Springer et al., 1998). Therefore, class I dimers that are not associated with the PLC may be either peptide-bound or peptide-free. To be able to look only at empty dimers, investigations have been done on cells that are deficient in TAP function, or in cells with chemically inhibited proteasomes (Baas et al., 2002; Day et al., 1995; Hsu et al., 1991; Paulsson et al., 2002; Pentcheva et al., 2001). These conditions cause peptide deficiency, during which class I molecules cannot proceed to the cell surface and remain EndoH sensitive. In addition, in such experiments, MHC class I molecules rapidly bind endogenous peptides that are added at the point of lysis (Townsend et al., 1989a). This would suggest that these molecules are peptide-free. However, it was shown that upon decreasing the temperature, peptide-bound murine class I molecules can be found at the cell surface in TAP-deficient cells. In such molecules, the peptide repertoire is less optimal (stabilizing against thermal
denaturation) than in wild type cells. Therefore, it seems that class I molecules in TAP-deficient cells bind no or low-affinity peptides, and thus they are termed peptide-receptive. Since MHC class I molecules do not need the components of the loading complex for peptide binding, but for peptide optimization, peptide-receptive forms may be either uncomplexed or associated with the PLC.

Figure 3: Retrieval model of MHC class I trafficking. Peptide-bound and peptide-receptive class I molecules can both be taken up into COPII vesicles and transported to the ERGIC and the Golgi apparatus, but peptide-receptive forms are returned from there to the ER in COPI vesicles (Figure courtesy of Patrycja Kozik and Sebastian Springer).

Peptide-receptive class I molecules are kept inside the cell, and are not exported to the cell surface. How is this achieved? Taking into account the experience from other model proteins of intracellular trafficking, there are two possibilities: either they are retained in the ER (retention model, see Figure 2), or they exit the endoplasmic reticulum and are transported to a later organelle of the
secretory pathway (the ERGIC or the cis-Golgi) from where they are returned to the ER (retrieval model, see Figure 3).

In the retention model, a protein cannot leave the ER because specific mechanisms prevent it from becoming packaged into COP II transport vesicles. This is true for many misfolded proteins as well as for some folded proteins like ribophorins (Fu and Kreibich, 2000; Nothurfft et al., 2000). Indeed, free heavy chains are complexed with the ER chaperone calnexin (Jackson et al., 1994), and HC-β2m dimers are usually bound to calreticulin or to the entire loading complex; these interactions with ER proteins may retain class I in the ER (Hendershot et al., 2000, Nehls et al., 2000). In addition, peptide-receptive class I molecules may be excluded from COPII transport vesicles because they lack an ER export signal (di-acidic sequence: DXE or DXD, di-hydrophobic motif: FF, YY, FY (Sevier et al., 2000; Nishimura and Balch, 1997)) which the COPII coat recognizes, and which they may only acquire upon peptide binding through association with a transport receptor (Antonny and Schekman, 2001; Herrmann et al., 1999). Thus, according to the retention model, vesicles that leave the ER do not contain any peptide-receptive class I molecules. In addition, components of the loading complex have no access to COPII vesicles.

In contrast to the retention model, the retrieval model postulates that all forms of MHC class I molecules can enter COPII vesicles, but that peptide-receptive forms of class I are recognized at a later stage and returned to the ER. Indeed, retrieval in COPI Golgi–to–ER vesicles enables the cycling of many proteins between the ER and the Golgi (Lippincott-Schwartz et al., 1989, Stornaiuolo et al., 2003). Accordingly, retrieval sequences on calreticulin
(–KDEL), and tapasin and calnexin (–KKXX) may direct these proteins into COPI vesicles at the ERGIC or the Golgi apparatus (Stornaiuolo et al., 2003). In this way, any class I molecules that do not bind high-affinity peptides may remain bound to these proteins and could then be retrieved to the ER. Even if only small amounts of heavy chain or dimers are found in the Golgi apparatus at steady state, this does not rule out a retrieval mechanism, since the efficiency of retrieval may be very high. The retrieval model therefore predicts that all forms of class I, plus possibly the proteins of the loading complex, have access to COPII vesicles and to the ERGIC and cis-Golgi, and that sorting between high affinity peptide-loaded class I and its peptide-receptive forms takes place in the ERGIC or the cis-Golgi.

Like often in biology, it is also possible that both models, retention and retrieval, are to some extent valid in the localization of class I inside the cell, and that they - for example - apply differentially to free heavy chains and peptide-receptive dimers of class I.

1.5. The generation of peptides and their binding to class I molecules

1.5.1. Peptide generation

Endogenous peptides are generated mostly in the cytosol by the proteasome. Proteasomal degradation supplies peptides of 3-22 amino acids with C-terminal residues that are often hydrophobic or basic, which is favored by both TAP and class I (Kisselev et al., 1999). In order to be transported to the endoplasmic reticulum, peptides are processed by cytosolic peptidases in the
cytosol; first by the puromycin-sensitive aminopeptidase and bleomycin hydrolase (Stoltze et al., 2000), next (below the length of 15 amino acids) by tripeptidyl peptidase II (Reits et al., 2004; Saveau et al., 2005). In the ER, the amino terminus of the peptides is further trimmed by aminopeptidases ERAP1 and ERAP2 (or their human ortholog, ERAAP) to an appropriate length for class I (Brooks et al., 2000a; Paz et al., 1999; Saric et al., 2002; Saveau et al., 2005; Serwold et al., 2002).

1.5.2. Peptide delivery to the ER

Peptides generated in the cytosol are delivered to the ER lumen by the TAP transporter. The efficient delivery of the peptides from the proteasome to TAP could be caused either by direct physical contact between the proteasome and TAP (Rivett, 1993), or by 'substrate channeling' of the peptides to TAP by heat shock proteins (Srivastava et al., 1994).

In order to be translocated into the ER by TAP, peptides need to fulfill certain size and sequence requirements.

1.5.2.1. Peptide size

Peptides up to 40 amino acids in length can be transported by TAP, but there is a decrease in the transport efficiency with the increase in peptide length (Heemels and Ploegh, 1994; Koopmann et al., 1996; Momburg et al., 1994). Peptides of 8-16 amino acids show equal binding affinities to TAP (Van Endert et al., 1994), but only peptides of 8-12 amino acids have equal transport efficiencies (Koopmann et al., 1996).
1.5.2.2. Peptide sequence

It was shown that for peptide binding to TAP, amino acids at positions 1-3 and at the C-terminus are important. The negative effect of proline at the position 3 is visible for peptide binding to mouse and human TAP (Neisig et al., 1995). Free amino and carboxy termini are also a strict requirement for peptide transport by TAP (Momburg et al., 1994; Schaumacher et al., 1994).

1.5.3. Peptide binding to class I

The peptides that are delivered to the ER by TAP bind to class I with different affinities. There are two important properties of the peptides that determine how strongly they bind to the class I HC-β₂m complexes: size and sequence.

1.5.3.1. Peptide size

Usually, peptides that are bound to class I molecules are eight to eleven amino acids in length, but longer peptides can also bind to MHC class I, since a 13mer was eluted from HLA-A2 (Guo et al., 1992; Henderson et al., 1992; Wei and Cresswell, 1992). The center of such long peptides buckles out of the peptide binding groove (Fremont et al., 1992; Guo et al., 1992; Madden et al., 1993), towards the T cell receptor, while the anchor residues and the termini are fixed (Falk et al., 1991; Guo et al., 1992; Hunt et al., 1992; Jardetzky et al., 1991).
1.5.3.2. Peptide sequence

1.5.3.2.1. Anchor residues

Peptide binding with high affinity to MHC class I molecules requires certain residues in the sequence of the peptide. These residues are called “anchor residues”, and they point downwards into the pockets of the binding groove (Rammensee, 1995).

For peptides that bind to human class I, anchor residues are located at positions 2 and ω (the last residue) (Bjorkman et al., 1987; Engelhard, 1994; Garrett et al., 1989; Guo et al., 1992; Madden et al., 1992; Madden et al., 1993; Saper et al., 1991; Silver et al., 1992).

Peptides that bind to mouse class I molecules have an anchor residue at the last position, and in addition to it, also in the center of the peptide, at the position ω-3 in H-2Kb, and at the position five for H-2Db (Fremont et al., 1992; Zhang et al., 1992; Young et al., 1994). H-2Kb binds phenylalanine or tyrosine at position ω-3 and leucine at the last position, and H-2Db binds asparagine at position five and methionine at position ω (Falk et al., 1991, Van Bleek and Nathenson, 1991).

In addition to the main anchor residues mentioned above, there are secondary anchor residues at position three, both in human and mouse (Young et al., 1994; Matsumura et al., 1992). However, they are not strictly required for the binding of the peptide to the class I molecules (Jardetzky et al., 1991).
1.5.3.2.2. N- and C-termini

In addition to anchor residues, the termini of the peptide are important for peptide binding to class I. The importance of the N- and C-termini of the peptide was shown by studies where N- or C-terminal residues were removed or modified (acetylated or amidated). The removal of the N-terminal amino acid in the peptide sequence decreased the binding affinity of the peptide to the class I molecule, but peptide-class I complexes were still formed (Bouvier and Wiley, 1994; Utz et al., 1992). Molecular dynamic simulation suggests that the region of the MHC class I binding groove where the N-terminus of peptide binds is rather rigid (Zacharias and Springer 2004). In a recently solved crystal structure, the absence of the N-terminus of the peptide does not change the structure of the binding site (Glithero et al., 2006; Khan et al., 2000).

The removal of the C-terminal residue in the peptide sequence dramatically reduces the peptide affinity to class I because both the C-terminal carboxylate group of the peptide and the C-terminal anchor residue are missing (Cerundolo et al., 1991). This was proposed by molecular dynamics simulation to be due to the fact that the respective region of the class I peptide binding groove is quite flexible, and that it is be stabilized by the peptide (Petrone and Garcia, 2004; Zacharias and Springer, 2004).
1.6. The role of the chaperone molecules in peptide optimization and trafficking of peptide-receptive class I molecules

After their synthesis, class I molecules require the help of chaperones (also called assembly factors) to fold, associate with β2m, and bind high-affinity peptide. The chaperone proteins also affect the intracellular localization of class I in a way that is not easy to differentiate experimentally from their influence on folding and peptide binding.

1.6.1. Calnexin

Calnexin (CNX) is 65 kDa transmembrane protein with a lectin domain in the ER lumen. It recognizes monoglucosylated substrates and helps them to fold properly (Hammond et al., 1994; Peterson et al., 1995). In addition to its glycan-dependent function, it may also directly recognize unfolded patches on proteins, since it interacts with non-glycosylated proteins too (Carreno et al., 1995; Ihara et al., 1999; Saito et al., 1999). Calnexin binding to monoglucosylated substrates is highly specific, while it binds to some unfolded proteins independent of their glucosylation status and suppresses their aggregation (Cannon et al., 1996; Danilczyk and Williams, 2001; Ihara et al., 1999; Leach et al., 2002;). Calnexin has been shown not to hydrolyze ATP in this process, unlike the major ER hsp70 chaperone, BiP.

Calnexin binds to free heavy chains of class I (Sadasivan et al., 1996; Solheim et al., 1997) but not to HC-β2m complexes (Diedrich et al., 2001; Noessner and Parham, 1995; Sadasivan et al., 1996; Sugita and Brenner, 1994; Tector and Salter, 1995; Tector et al., 1997; Zhang et al., 1995).
Studies on human calnexin-deficient cells have shown that calnexin is not required for assembly, transport and cell surface expression of human MHC class I molecules (Prasad et al., 1998; Sadasivan et al., 1995; Scott and Dawson 1995). In contrast, the deletion of mouse calnexin causes class I to misfold and aggregate (Vassilakos et al., 1996).

One known function of calnexin in the maturation of class I molecules is to recruit the thiol oxidoreductase ERp57 in a molar ratio of 1:1 (Zapun et al., 1998) in order to support, by the formation of correct disulfide bonds, the proper folding of the class I heavy chain into a conformation that allows binding of β2m (Degen et al., 1992; Farmery et al., 2000; Lindquist et al., 2001; Noessner and Parham, 1995; Tector and Salter, 1995; Tector et al., 1997). The CNX-ERp57 complex is formed before the class I heavy chain binds to it (Diedrich et al., 2001).

In addition to its function in the folding of MHC class I heavy chains, calnexin also is required for the targeting of misfolded class I heavy chains for ER-associated degradation, as is the case for other glycoproteins (Liu et al., 1997; Wiertz et al., 1996).

In addition, calnexin may play a role in the retention of the free heavy chains of class I molecules in the endoplasmic reticulum due to the KXRRRX sequence on its C-terminus, which is an ER localization signal in both human and mouse calnexin (Ellgard and Helenius, 2003; Hammond and Helenius, 1994; Jackson et al., 1994).
1.6.2. Calreticulin

Calreticulin is a 46 kDa soluble lectin, which, like calnexin (to the lumenal domain of which it is very similar), helps in the proper folding of monoglucosylated proteins; it also prevents aggregation of unfolded proteins (Hammond et al., 1994; Leach et al., 2002; Peterson et al., 1995). Calreticulin binds to monoglucosylated class I (but not when three glucose residues are present on the MHC class I molecule (Sadasivan et al., 1996)), replacing calnexin in the class I maturation process. Calreticulin binds to HC-β2m complexes, but not or only poorly to free heavy chains (Sadasivan et al., 1996; Solheim et al., 1997). A thermal aggregation assay has shown that calreticulin forms high molecular weight complexes with the human allele, HLA-A2, at 50°C, but not at 37°C - this suggests a role in binding to misfolded proteins. Calreticulin may also help to stabilize HC-β2m dimers (Mancino et al., 2002).

Studies on calreticulin-deficient cells have shown that calreticulin is not critical for the formation of the peptide loading complex since in its absence, a loading complex of TAP, tapasin, ERp57, and class I is still formed. Also, the incorporation of the HC-β2m dimers into the loading complex does not require calreticulin (Gao et al., 2002).

The deletion of calreticulin results in the decrease by 50-70% of cell surface expression of K\(^b\) and D\(^b\) alleles of class I, and in their accelerated export out of the ER (Gao et al., 2002). This suggests that in these cells, peptide loading is inefficient but class I is nevertheless transported to the surface bound to low-affinity peptides which then dissociate, and that surface class I is unstable and rapidly endocytosed. This suggests that calreticulin helps
to retain HC-β2m complexes in the ER (or retrieve them from the ERGIC or cis-Golgi) for peptide optimization (Lewis et al., 1998). According to this model, calreticulin itself may not itself act in peptide optimization.

In summary, calreticulin as a lectin acts in the proper folding of the class I dimers, but it may also have an impact on the class I intracellular localization.

1.6.3. ERp57

ERp57 (ER protein of 57 kDa, also called ER-60) is a member of the protein disulfide isomerase (PDI) family. It contains two thioredoxin motifs (Hirano et al., 1995; Srivastava 1991). Thioredoxins contain the sequence CGHC, which plays a role in disulfide bond oxidation/reduction, at positions 57/60 and 406/409, respectively (Zapun et al., 1998). The N-terminal cysteine of the thioredoxin motif interacts with the protein substrate and acts in the formation of the disulfide bonds, whereas the C-terminal cysteine has a function in a so-called “escape mechanism”, forming a disulfide bond with the N-terminal cysteine and thus releasing it from a mixed disulfide intermediate that is otherwise slow to dissolve, for example with a terminally unfolded protein (Walker and Gilbert, 1997).

ERp57 interacts specifically with monoglucosylated substrates (Oliver et al., 1996; Oliver et al., 1997), and there is a significant reduction in its association with a glycoprotein when the trimming of the ligand oligosaccharide to the monoglucosylated form is prevented. Since ERp57 does not bind to sugars itself, it requires lectins to recognize its substrates. It
interacts with calnexin or calreticulin to assist in the folding of N-glycosylated proteins (Oliver et al., 1997; Oliver et al., 1999; Zapun et al., 1998).

ERp57 is important at two steps of class I maturation (Hughes and Cresswell, 1998; Lindquist et al., 1998; Morrice and Powis, 1998). First, together with calnexin, it plays a role in the proper folding of the heavy chain. Second, it is present in the loading complex and bridges tapasin and calreticulin (Cresswell et al., 1999; Lindquist et al., 2001).

When it acts in the proper folding of the class I heavy chain, ERp57 is involved in the formation of disulfide bonds in α2 (between residues Cys101 and Cys164, in the peptide binding domain) and α3 domains (between residues Cys203 and Cys259) (Elliott et al., 1997; Farmery et al., 2000; Helenius and Aebi, 2001; Tector and Salter, 1995) that are crucial for the cell surface expression of class I (Miyazaki et al., 1986b; Smith et al., 1995; Warburton et al., 1994).

As a component of the loading complex, ERp57, together with tapasin, plays a role in the optimization of peptides that are bound to class I (Dick et al., 2002). In experiments where the formation of the tapasin-ERp57 complex was inhibited, peptide loading on class I was suboptimal (Dick et al., 2002, Creswell 2003). In ERp57-deficient cells, there is a significant reduction of the cell surface expression of class I. In addition, there is a decrease in the association of HC-β2m complexes with the loading complex. These data suggest that ERp57 retains class I in the PLC until it binds high affinity peptide (Garbi et al., 2006).
What is the role of the thiol oxidoreductase activity in class I peptide loading? One explanation is that ERp57 forms a disulfide conjugate with tapasin (Dick et al., 2002) and thus bridges it to calreticulin. An alternative explanation maintains that the disulfide bond in the $\alpha_2$ domain of the class I heavy chain is broken and reformed during peptide exchange. When high affinity peptide is bound to class I, this may cause the formation of a stable disulfide bond and the release of the class I molecule from the loading complex (Dick et al., 2002). Indeed, there are conformational changes in class I molecules upon peptide binding (Rigney et al., 1998; Smith et al., 1995, Springer et al., 1998), but it has not been decisively clarified whether disulfide isomerisation of class I actually occurs during peptide binding in the cell (Park et al., 2006).

In summary, ERp57 acts in the maturation of class I molecules and helps them to bind optimal peptides. This thiol oxidoreductase influences the class I export to the cell surface, and it could also play a role in the intracellular trafficking of the HC-$\beta_2$m complexes.

Recently, there has been a report of another protein disulfide isomerase, PDI, in the loading complex (Park et al., 2006). Ahn and co-workers have shown that PDI regulates the redox status of MHC class I molecules during peptide loading and contributes to the antigen presentation at the cell surface. In addition, in ERp57-deficient cells, class I heavy chain disulfides are in an oxidized state which suggests that an additional factor controls HC oxidation (Garbi et al., 2006). This additional factor might be PDI, but its exact mechanism of action on class I remains unknown. While ERp57 acts
specifically on glycoproteins in association with calnexin or calreticulin, PDI does not have the same specificity towards glycoproteins, and works either alone (Kulp et al., 2006), or in association with BiP (Mayer et al., 2000).

1.6.4. The Transporter associated with antigen processing (TAP)

TAP is a member of the ABC (ATP binding cassette) family of transporters. It consists of two subunits, TAP1 (81 kDa) and TAP2 (75 kDa) (Powis et al., 1991; Spies and DeMars, 1991; Trowsdale et al., 1990). TAP1 is stable in cells that lack TAP2 and can associate with class I, but for peptide translocation into the ER, both subunits are required (Androlewicz et al., 1994, Powis et al., 1991; Spies and DeMars, 1991). Each subunit contains two domains: the hydrophobic transmembrane domain (TMD), which consists of six alpha helices, and the cytosolic nucleotide binding domain (NBD). The TMDs bind the peptides and forms a translocation channel within the ER membrane, while the NBDs supply the energy for peptide translocation by ATP hydrolysis (Gorbulev et al., 2001).

Usually, HC-β2m complexes in TAP-deficient cells do not bind high-affinity peptides and are not exported to the cell surface (Cerundolo et al., 1990; van Kaer et al., 1992). However, there are some MHC class I molecules that do not interact with the PLC (e.g., HLA-B*1518) and still proceed to the cell surface. However, for these alleles, there are usually many more peptide-receptive molecules at the cell surface than for those that do interact with TAP (Turnquist et al., 2000). Another interesting observation is that lowering the temperature helps surface export of mouse class I alleles in the absence of TAP (Ljunggren et al., 1990; Ploegh 1990; Van Kaer et al., 1992).
TAP localizes mostly to the lumen of the ER, and in addition, there is some colocalization with p53 (an ERGIC/cis-Golgi marker) (Kleijmeer et al., 1992). This, and the fact that TAP does not posses any retention signal, suggests that TAP may be loaded together with class I into COPII vesicles and transported to the ERGIC or the cis-Golgi, and then become recycled to the ER.

1.6.5. Tapasin

Tapasin (TPN) is a 48 kDa transmembrane protein, the most important in the peptide loading complex. In the absence of tapasin, the loading complex is not formed (Ortmann et al., 1997).

Tapasin was proposed to interact with two regions of the class I heavy chain: one within the α2 domain, between residues 122 and 136, and the second in the α3 domain between residues 227 and 229 (Lewis et al., 2002; Paquet et al., 2002; Suh et al., 1999; Turnquist et al., 2001; Yu et al., 1999).

In cells that lack tapasin, most class I molecules cannot proceed to the cell surface. They do not reach the medial Golgi since they remain sensitive to endoglycosidase H (EndoH) digestion (Zernich et al., 2005). The mutant of HLA-A2, T134K, which does not bind to tapasin, can still be exported to the cell surface, but with sub-optimal peptides (Lewis et al., 1996; Peace-Brewer et al., 1996). The same was observed for the mouse alleles, H-2D^b and H-2K^b (Garbi et al., 2000; Grandea et al., 2000). In the absence of tapasin, most class I molecules, for example HLA-B^*4402, cannot bind high-affinity peptides. In contrast, other class I molecules do not require functional tapasin for peptide
binding, for example HLA-B*4405, but the repertoire of the peptides bound to these molecules in the absence of tapasin may be different. Tapasin helps MHC class I molecules to optimize their peptides over time (Williams et al., 2002). The molecular mechanism of tapasin action on class I remains unknown.

Tapasin, not calreticulin, recruits ERp57 to the loading complex. In tapasin-deficient cells, the amount of ERp57 in the cell is decreased (Hughes and Cresswell, 1998), and ERp57 is not found in the peptide loading complex (Dick et al., 2002), while in calreticulin-deficient cells, ERp57 still associates with the PLC (Gao et al., 2002). Also, in the absence of TAP, the peptide loading complex is still formed from tapasin, ERp57, and calreticulin (Sadasivan et al., 1996).

Tapasin interacts with ERp57 through a disulfide bond between Cys7 of tapasin and Cys95 of ERp57. This interaction increases the overall coherence of the loading complex, since now ERp57 is bound to class I in two ways: through tapasin and through calreticulin (Dick et al., 2002).

By the formation of a complex with ERp57, tapasin also exerts influence on calreticulin: in tapasin-deficient cells, the amount of calreticulin in the peptide loading complex is reduced (Solheim et al., 1997). However, the binding of calreticulin to the HC-β2m complex is independent of tapasin (Paquet and Williams, 2002; Turnquist et al., 2002).

Tapasin stabilizes TAP at least in some cell lines, increasing its steady-state level (Bangia et al., 1999; Garbi et al., 2003, Lehner et al., 1998). This results in a higher amount of peptides transported into the endoplasmic reticulum (Lehner et al., 1998; Li et al., 2000) and perhaps also increases the
amount of the peptide in the proximity of class I HC-β2m complexes, enhancing peptide loading onto class I (Ortmann et al., 1994, Suh et al., 1994). The comparison of a soluble form of tapasin (which does not interact with TAP) to the wild type protein has shown that the rate of the peptide optimization by the soluble form is reduced, as compared to the effect of the full length protein (Williams et al., 2002). In tapasin-deficient cells, there is no association of TAP with class I, but the impaired peptide binding and reduced cell surface expression of class I is rather due to the absence of the tapasin from the loading complex than due to the absence of TAP-class I association (Grandea et al., 1995). The physical contact between the TAP and class I may increase the local peptide concentration, and therefore more peptides may be available to bind to MHC class I, but TAP is not required for peptide fragments to bind to class I (Wei and Cresswell, 1992; Snyder et al., 1994).

Tapasin was shown to interact with the COPI coat (Paulsson et al., 2002), which is probably due to its C-terminal KKXX signal. This interaction may mediate the retrieval of peptide-receptive forms of class I molecules from the ERGIC and/or cis-Golgi, since non-optimally loaded HC-β2m complexes were found associated with COPI coat proteins in the presence of tapasin, but not in its absence (Paulson et al., 2002). In contradiction, Edidin and co-workers have shown that GFP-tapasin is retained in the ER, and has no access to the ER exit sites (Pentcheva et al., 2001). Indeed, the intracellular localization of tapasin remains unclear, and the respective contributions of calreticulin and tapasin to the intracellular retention of peptide-receptive class I molecules have not been satisfactorily evaluated.
A close homolog of tapasin, TAPBP-R, has been described (Teng et al., 2002), but it is unknown whether it has a function in the loading complex, or in antigen presentation.

1.7. The aim of this thesis

The aim of this thesis was to differentiate between the different localization models that exist for peptide-receptive class I and to elucidate to define the respective mechanisms. To address these questions, I have observed the peptide-bound and peptide-receptive forms of class I molecules in cells with fluorescence microscopy.
2. Materials and methods

2.1. Materials

2.1.1. Cell lines

CHO (Chinese Hamster Ovary) and TAP-deficient CHO (TAP2d) fibroblasts, kindly provided by K. Gould (London), were grown in 75 cm² cell culture dishes in Ham’s F12 medium supplemented with 5% FCS and PSG (100 U/ml penicillin, 100 µg/ml streptomycin, and 2 mM glutamine). Vero (African green monkey kidney) cells were grown in 75 cm² cell culture dishes in DMEM medium, supplemented with 10% FCS and PSG. β2m-negative fibroblasts (β2m-NF) and stable β2m-NF H-2Kb-GFP, kindly provided by M.Edidin (Spiliotis et al., 2002), were grown in 75 cm² cell culture dishes in DMEM medium, supplemented with 10% FCS and PSG. To maintain the expression of H-2Kb-GFP in the stable cell line, 200 µg/ml G418 was added to the culture medium. Wild type mouse ear fibroblasts (MEF) and tapasin⁺/⁻ MEF, kindly provided by L. van Kaer (Grandea et al., 2000), were grown in 75 cm² cell culture dishes in DMEM medium, supplemented with 10% FCS and PSG. K41 and K42 cells, kindly provided by Tim Elliott (Gao et al., 2002), were grown in 75 cm² cell culture dishes in RPMI 1640 medium, supplemented with 10% FCS and PSG. Human T1 and T2 lymphoblastoid cell lines, a kind gift from A. Townsend (Oxford, UK), were maintained in 75 cm² cell culture dishes in RPMI 1640 medium, supplemented with 10% FCS and PSG. All cell lines were grown at 37 °C in an atmosphere of 5% CO₂.
2.1.2. Fluorescent constructs

**Table 1.** Fluorescent constructs used in colocalization experiments

<table>
<thead>
<tr>
<th>Fluorescent construct</th>
<th>Target organelle</th>
<th>Source</th>
<th>Amount for transfection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sec22-CFP</td>
<td>Endoplasmic reticulum and ER exit sites</td>
<td>I. Majoul (London)</td>
<td>20 µg</td>
</tr>
<tr>
<td>Sec22-YFP</td>
<td>Endoplasmic reticulum and ER exit sites</td>
<td>I. Majoul (London)</td>
<td>20 µg</td>
</tr>
<tr>
<td>p23-CFP</td>
<td>cis-Golgi</td>
<td>I. Majoul (London)</td>
<td>20 µg</td>
</tr>
<tr>
<td>GalT-CFP</td>
<td>trans-Golgi</td>
<td>J. Lippincott-Schwartz (Bethesda, MD, USA)</td>
<td>20 µg</td>
</tr>
<tr>
<td>GalT-YFP</td>
<td>trans-Golgi</td>
<td>J. Lippincott-Schwartz (Bethesda, MD, USA)</td>
<td>20 µg</td>
</tr>
<tr>
<td>CD63-CFP</td>
<td>lysosomes</td>
<td>P. Luzio (Cambridge)</td>
<td>20 µg</td>
</tr>
<tr>
<td>CD63-YFP</td>
<td>lysosomes</td>
<td>P. Luzio (Cambridge)</td>
<td>20 µg</td>
</tr>
</tbody>
</table>
### 2.1.3. Antibodies

#### Table 2. Antibodies against organelle markers

<table>
<thead>
<tr>
<th>Primary Antibody</th>
<th>Target organelle</th>
<th>Source</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>rabbit anti-calnexin</td>
<td>Endoplasmic reticulum (ER)</td>
<td>D. Williams (Toronto)</td>
<td>1:200</td>
</tr>
<tr>
<td>rabbit anti-Sec31</td>
<td>ER exit sites</td>
<td>F. Gorelick (Yale)</td>
<td>1:200</td>
</tr>
<tr>
<td>rabbit anti-p58</td>
<td>ER-Golgi intermediate compartment (ERGIC)</td>
<td>R. Peterson (Stockholm)</td>
<td>1:200</td>
</tr>
</tbody>
</table>

#### Table 3. Antibodies against class I

<table>
<thead>
<tr>
<th>Primary Antibody</th>
<th>Source</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>mouse anti-HLA-B and -C (HC10)</td>
<td>A. Townsend (Oxford)</td>
<td>1:200</td>
</tr>
<tr>
<td>rabbit anti-H-2D(^b) (T18)</td>
<td>T. Elliott (Southampton)</td>
<td>1:200</td>
</tr>
</tbody>
</table>

#### Table 4. Secondary antibodies

<table>
<thead>
<tr>
<th>Secondary Antibody</th>
<th>Source</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>goat anti-rabbit-Cy2</td>
<td>Jackson ImmunoResearch Laboratories (Soham, UK).</td>
<td>1:200</td>
</tr>
<tr>
<td>goat anti-mouse-Cy2</td>
<td>Jackson ImmunoResearch Laboratories (Soham, UK).</td>
<td>1:200</td>
</tr>
<tr>
<td>goat anti-rabbit-Cy3</td>
<td>Jackson ImmunoResearch Laboratories (Soham, UK).</td>
<td>1:200</td>
</tr>
<tr>
<td>goat anti-mouse-Cy3</td>
<td>Jackson ImmunoResearch Laboratories (Soham, UK).</td>
<td>1:200</td>
</tr>
<tr>
<td>goat anti-rabbit-Cy5</td>
<td>Jackson ImmunoResearch Laboratories (Soham, UK).</td>
<td>1:200</td>
</tr>
<tr>
<td>goat anti-mouse-Cy5</td>
<td>Jackson ImmunoResearch Laboratories (Soham, UK).</td>
<td>1:200</td>
</tr>
</tbody>
</table>
2.1.4. Pure peptides

SIINFEKL (from ovalbumin, amino acids 257-264; H-2D\textsuperscript{b} and H-2K\textsuperscript{b}) and ILKEPVGHV (from HIV pol 476-484, HLA-A*0201), purified by HPLC, were from Biosyntan (Berlin, Germany). FAPGNYPAL (Sendai virus nucleoprotein, 324-332, H-2D\textsuperscript{b}) and its derivatives FAPGNYPAA, FAPGNYPA, FAPGNYP, FAPGNY, FAPGN, and NYPAL were obtained from T. Elliott.
2.2. Methods

2.2.1. Generation of expression plasmids

2.2.1.1. Amplification of H-2K<sup>b</sup> and H-2D<sup>b</sup> cDNA

A cDNA-encoding wild-type H-2K<sup>b</sup> and H-2D<sup>b</sup> was obtained from T.Elliott (Southampton). cDNA was amplified by the polymerase chain reaction with a mutagenic 5’ forward primer containing a SalI site (H-2K<sup>b</sup>: CCC CGT CGA CCA TGG TAC CGT GCA CGC TG, H-2D<sup>b</sup>: CCC CGT CGA CCA TGG GGG CGA TGG CTC CG) and a reverse 3’ primer which replaces the stop codon with a BamHI restriction site (H-2K<sup>b</sup>: GTG GAT CCG CTA GAG GAT GGT CA, H-2D<sup>b</sup>: GTG GAT CCG CTT TAC AAT CTC GGA GAG A). Each 5 µl PCR sample contained forward and reverse primer pairs (final concentration of each primer: 1 µM), dNTP mix, Pfu DNA polymerase, 1 x buffer containing MgCl<sub>2</sub> (Fermentas) and cDNA template. Each PCR reaction consisted of initial denaturation (10 minutes, 95 °C), and then 30 cycles of denaturation (1 minute, 95 °C), annealing (1 minute, 65 °C), extension (1 minute, 75 °C). PCR reaction was finished by final extension (5 minute, 75 °C). PCR products were analyzed by separation on the 1% agarose gel for 30 minutes and 100 V in an *i-mupid* Mini Cel electrophoresis unit (Cosmo Bio Co. Ltd., Tokyo Japan) using 0.5 x TAE buffer (0.5 mM EDTA, 20 mM Tris-HCl, pH 8.0). PCR product should be of about 1000 bp for H-2K<sup>b</sup> and 1100 bp for H-2D<sup>b</sup>.
2.2.1.2. Restriction digest and ligation of H-2K\textsuperscript{b} and H-2D\textsuperscript{b} inserts and pEGFP-N1 and Ds-Red-N1 vectors

H-2K\textsuperscript{b} and H-2D\textsuperscript{b} primers were designed such that two distinct restriction sites, i.e. a Sall restriction at the 5’- and a BamHI restriction site at the 3’-end, were amplified into H-2K\textsuperscript{b} and H-2D\textsuperscript{b} sequence. The pEGFP-N1 and Ds-Red-N1 expression vectors used for cloning (Clontech Laboratories Inc., Heidelberg, Germany) possess the same restriction sites, and therefore Sall/BamHI double digestion was used for preparation of both insert and vector. Each digestion sample contained 26 U of BamHI (MBI Fermentas), 54 U of Sall (MBI Fermentas), 1x BamHI buffer. From the peGFP-N1 and Ds-Red-N1 plasmids, 13 µg were used, whereas 49 µl of amplified H-2K\textsuperscript{b} and H-2D\textsuperscript{b} (PCR) products were used for double digestion. Digestion was carried out overnight at 37 °C. Digested vector DNA was separated on 0.7% agarose gel and a fragment of the correct size (i.e. about 4700 bp) was purified from the gel using QIAquick Gel Elution Kit (QIAGEN) and resuspended in 20 µl of sterile ddH\textsubscript{2}O. The digested insert was purified by QIAquick Gel Elution Kit (QIAGEN) and also resuspended in 20 µl of sterile ddH\textsubscript{2}O.

For the ligation of the digested insert into the linearized vector, a three-fold molar excess of the insert compared to the vector was used. Further, 2U of T4 DNA ligase (MBI Fermentas) and 1x T4 ligase buffer were added to the ligation reaction. The ligation, the total volume of which was 10 µl, was carried out at room temperature for 3 hours.
2.2.1.3. Preparation of chemically competent *E.Coli* cells for transformation

Frozen *E.coli* XL1 stock culture was thawed on ice, plated onto LB-agar plates, and incubated overnight at 37 °C. Colonies from LB plates were used to inoculate 3 ml of LB liquid culture which was again incubated overnight at 37 °C. The culture was used to inoculate of 100 ml of LB medium which was then grown at 37 °C until the suspension reached an optical density of OD₆₀₀ = 0.6. Then, the bacterial suspension was cooled on ice for 10 minutes, transferred into two 50 ml Falcon tubes, and centrifuged at 5000 x g, 7 minutes, 4 °C. The supernatants were then discarded, and the pellets were resuspended and combined in 30 ml of sterile 0.1 M MgCl₂ by pipetting. After centrifugation at 3000 x g, 10 minutes, 4 °C, the supernatant was discarded, and the pellet was resuspended in 30 ml of sterile 0.1 M CaCl₂ and incubated on ice for 20 minutes. The bacterial suspension was then centrifuged at 3000 x g, 10 minutes, 4 °C, the supernatant was discarded, and the pellet was resuspended in 5 ml of sterile 0.1 M CaCl₂ + 15% glycerol. The bacterial suspension was aliquotted into 1.5 ml sterile microcentrifuge tubes (150 µl to each tube), frozen in liquid nitrogen, and stored at -80 °C.

2.2.1.4. Transformation of competent cells

One aliquot of competent *E.Coli* XL1 cells was mixed with 5 µl of ligation mixture and incubated for 5 minutes on ice. Next, the bacterial suspension was subjected to a heat shock by transferring it to a 42 °C water
bath for 90 seconds. Next, 1 ml of LB medium was added to the bacterial suspension, and the sample was incubated at 37 °C for 1 hour. After this time, transformed bacteria were plated on LB agar plates containing 25 µg/ml kanamycin (Applichem) as selective antibiotic and cultured overnight at 37 °C.

2.2.1.5. Diagnostic digest

Clones from LB-Kanamycin plates were used for the inoculation of LB-Kanamycin liquid culture and incubated overnight at 37 °C. Next day, a plasmid mini-preparation was carried out in order to test for the correctness of the ligation. 1.5 ml of bacterial suspension was centrifuged at 7000 x g for 5 minutes at room temperature, supernatants were discarded and pellets were resuspended in 110 µl of solution I (50 mM glucose, 10 mM EDTA, pH 8.0, 25 mM Tris-HCl, pH 8.0). Next, 220 µl of solution II (0.2 N NaOH, 1% SDS) was added, and the bacterial suspension was incubated for 1 minute on ice. Finally, 165 µl of solution III (3M potassium acetate, 11.5% glacial acetic acid) was added, the mixture was vortexed, incubated 5 minutes on ice, and centrifuged at 15000 x g, 5 minutes at 4 °C. The supernatant was transferred to a 1.5 ml microcentrifuge tube containing 1 ml of 100% ethanol. Samples were incubated for 5 minutes on ice, and the DNA was pelleted by centrifugation at 15000 x g, 5 minutes, 4 °C. The DNA was next resuspended in 50 µl of TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) and subjected to RNAse A treatment (0.2 mg/ml) for 5 minutes at 42 °C. After vortexing the sample, an equal amount of phenol/chloroform was added. Next, the sample was vortexed again and
centrifuged at 16000 x g, 1 minute at room temperature. The aqueous phase was transferred to a fresh tube, 1/10 of the volume of 5M ammonium acetate and two volumes of ethanol were added, and the sample was incubated at -20 °C for 15 minutes. The DNA was pelleted by centrifugation at 16000 x g, 15 minutes, 4 °C. Next, the DNA pellet was resuspended in 20 µl of TE buffer. 1 µl of DNA solution was used for restriction analysis with SalI and BamHI enzymes to test the integration of the insert into the vector. The restriction digest was visualized by agarose gel electrophoresis. If the diagnostic digest revealed a clone to be positive, 5 µl of plasmid DNA were sent for sequence analysis.

2.2.1.6. Maxi-preparation of plasmids

Maxipreparation was done using QIAGEN maxi-prep kit according to a modified protocol of the supplier.

Colonies from LB-Kanamycin plates were used for the inoculation of 3 ml of LB-Kanamycin liquid culture and incubated overnight at 37 °C. The culture was then used for the inoculation of 250 ml of LB-Kanamycin medium and grown at 37 °C for next 16-18 hours. Cells were harvested by centrifugation at 3200 x g, 20 minutes, 4 °C. The pellet was resuspended in 10 ml of buffer P1 (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 100 µl/ml RNase A). Next, 10 ml of buffer P2 (200 mM NaOH, 1% SDS) was added and the sample was inverted 4-6 times and incubated for 5 minutes at room temperature. 10 ml of buffer P3 (3 M potassium acetate, pH 5.5) were then added, the sample was mixed thoroughly, transferred to a 50 ml Falcon tube, and centrifuged at 15000 x g, 20 minutes, 4 °C. The
supernatant was transferred to a new 50 ml Falcon tube, 2.5 ml of buffer ER were added, and the sample was inverted few times in order to mix and incubated on ice for 30 minutes. During this time, a QIAGEN tip-500 was equilibrated with 10 ml of buffer QBT (750 mM NaCl, 50 mM MOPS, pH 7.0, 15% isopropanol, 0.15% Triton X-100). The filtered lysate was applied to the QIAGEN tip-500, and the flowthrough was collected. The column was washed twice with 30 ml of buffer QC (1 M NaCl, 50 mM MOPS, pH 7.0, 15% isopropanol) and the DNA was eluted with 15 ml of buffer QN (1.6 M NaCl, 50 mM MOPS, pH 7.0, 15% isopropanol). The eluted DNA was kept on ice. Column was regenerated with 30 ml of sterile ddH$_2$O and equilibrated with 10 ml of buffer QBT. The first flowthrough was reapplied to the column, and the second flowthrough was collected. Column was washed twice with 30 ml of buffer QC, and the DNA was eluted with 15 ml of buffer QN. The DNA obtained after the second elution was combined with DNA from first elution and kept on ice. Column was regenerated with 30 ml of sterile ddH$_2$O and equilibrated with 10 ml of buffer QBT. The second flowthrough was reapplied to the column, and the fourth flowthrough was discarded. The column was washed twice with 30 ml of buffer QC, and the DNA was eluted with 15 ml of buffer QN. Eluted DNA was pooled with the DNA from the first and second elutions, and 31.5 ml of isopropanol were added to the combined fractions. The DNA was pelleted by centrifugation at 15000 x g, 30 minutes, 4 °C. The pellet was then transferred to a 2 ml microcentrifuge tube, washed with 300 µl of 70% ethanol, and centrifuged at 15000 x g, 5 minutes, 4 °C. The
supernatant was discarded, and the pellet was washed with 1 ml of 70% ethanol and centrifuged at 15000 x g, 5 minutes, 4 °C. The supernatant was discarded, and the pellet was air-dried for 5 minutes. After this time, the pellet was resuspended in 500 µl of TE buffer and the DNA concentration was measured in the Nanodrop spectrophotometer.

2.2.2. Transfections

The transfections of CHO, CHO TAP2d, Vero, β2m-NF, MEF, tapasin⁻/⁻ MEF, K41, K42, T1, and T2 cells were performed by electroporation following (Majoul et al., 2001). For transfection, cells were washed twice with PBS (1.5 mM KH₂PO₄, 2.5 mM KCl, 10 mM Na₂HPO₄, 130 mM NaCl. TBS (Tris buffered saline) is 10 mM Tris-Cl pH 7.5, 150 mM NaCl) and transferred into transfection medium (120 mM KCl, 10 mM KH₂PO₄, 5 mM MgCl₂, 0.15 mM CaCl₂, 25 mM HEPES, 2 mM EGTA, 2 mM ATP, and 5 mM oxidized glutatione). About 1-2 million cells were transfected in 200 µl final volume with 20 µg of maxiprep DNA (Qiagen, Hilden, Germany). CHO, TAP2d, Vero, β₂m-NF, MEF wt, tapasin⁻/⁻ MEF, K41 and K42 cells were transfected by electroporation at 500 V, 200 µs. T1 and T2 cells were transfected at 240 V, 40 µs.
2.2.3. Immunofluorescence microscopy

2.2.3.1. Colocalization experiments

2.2.3.1.1. Preparation of cells

2.2.3.1.1.1. Preparation of suspension cells

Cover slips (22 x 22 mm) were coated with 100 µl of 0.1% L-polylysine (Sigma) for 10 minutes. Then they were washed three times with 100 µl of sterile ddH₂O and dried. T1 and T2 cells were centrifuged at 200 x g, 5 minutes at room temperature, and 0.4 million cells were resuspended in 100 µl of PBS and seeded on the L-polylysine-coated coverslips for 45 min at 37 °C. Cells were then fixed with 4% paraformaldehyde in PBS for 30 min at 37 °C. After three washing steps (5 minutes each) using PBS, cells were permeabilized for 5 min with 0.1% Triton X–100 (Applichem) and washed again with PBS. T1 and T2 cells were then incubated with HC10 antibody in a moisturized chamber for one hour at room temperature in the presence of blocking medium (0.1% BSA in PBS) for primary, and (after three washings with PBS) for 30 minutes for fluorescently labeled secondary antibody for 30 min at room temperature. Cells were washed then three times with PBS and mounted on the microscope slides with Mowiol containing 50 mg/ml DABCO (Sigma-Aldrich).
For colocalization experiments, cells were either incubated with an antibody specific for the organelle marker protein (anti-calnexin, anti-Sec31 or anti-p58 antibody) in addition to HC10 antibody or transfected with 20 µg of DNA encoding Sec22-CFP/YFP, p23-CFP, GalT-CFP/YFP or CD63-CFP/YFP proteins 24 hours before fixation and staining with HC10 antibody.

2.2.3.1.2. Preparation of adherent cells

CHO, CHO TAP2d, Vero, β2m-NF, MEF, tapasin−/− MEF, K41, and K42 cells were transiently transfected with 20 µg of DNA encoding H-2Kb-GFP or H-2Db-GFP, spotted onto glass microscope slides in culture medium, and incubated for 24 h. The cells were then observed directly or washed with PBS, fixed with 4% paraformaldehyde in PBS for 30 min at room temperature, washed three times, and mounted onto microscope slides. For colocalization experiments, cells were either co-transfected with 20 µg of DNA encoding Sec22-CFP/YFP, p23-CFP, GalT-CFP/YFP or CD63-CFP/YFP proteins or stained with an antibody specific for the organelle marker protein (anti-calnexin, anti-Sec31 or anti-p58 antibody). For staining with antibody, cells were fixed with 4% PFA, washed three times with PBS, permeabilized for 5 min with 0.1% Triton X-100 and after next three washing steps, incubated with an antibody in a moisture chamber. After three washing steps with PBS, cells were incubated with secondary antibody, then washed
again and mounted onto the microscope slides with Moviol and DABCO.

### 2.2.3.1.2. Blocking protein transport in organelles

To arrest trafficking of class I molecules in the ERGIC, cells were incubated for 2 hours at 15 °C and then fixed with 4% paraformaldehyde in PBS for 30 min at 15 °C (Lippincott-Schwartz et al., 1990). To accumulate class I molecules in the Golgi apparatus, cells were incubated for 2 hours at 20 °C (Matlin et al, 1983) and then fixed with 4% paraformaldehyde in PBS for 30 min at 20 °C. The fixation was followed by washing the cells three times with PBS and either mounting them onto microscope slides or incubating them with antibodies.

### 2.2.3.2. Electroporation of the peptide

CHO, CHO TAP2d, Vero, β2m-NF, MEF, tapasin−/− MEF, K41, and K42 cells were washed twice in PBS and transferred to the transfection medium, then transiently transfected at 500 V and 200 µs with 20 µg of H-2K<sup>b</sup>-GFP or H-2D<sup>b</sup>-GFP maxiprep DNA (Qiagen), spotted onto glass microscope slides in the appropriate medium containing 100 nM or 300 nM of respective peptide, and incubated for 24 hours. After that time, cells were observed directly or fixed with 4% PFA, washed three times with PBS and mounted on the microscope slides prior to the visualization. For each peptide, 100 cells were observed and each cell was classified as either 0 or 1 depending on the presence of the class I-GFP molecules at its surface. Then the percentage of the cell surface expression of class I-GFP molecules was
calculated as the number of cells with cell surface expression of class I-GFP over the total number of the cell. This experiment was repeated seven times for two peptide concentrations: 100 nM and 300 nM. For each peptide and each peptide concentration, the mean value over the seven experiments was calculated according to the formula

\[ \bar{x} = \frac{1}{N} \sum_{i=1}^{N} x_i \]

\( \bar{x} \) - mean of \( X \), \( X \in \{x_1, ..., x_N\} \)

\( N \) - number of samples taken

For each mean value, the standard error of the mean was calculated according to the formula

\[ \sigma_{EM} = \frac{\sigma}{\sqrt{N}} \]

where:

\( \sigma_{EM} \) - standard error of the mean

\( \sigma \) - standard deviation of the random variable \( x \)

\[ \sigma = \sqrt{\frac{1}{N} \sum_{i=1}^{N} (x_i - \bar{x})^2} \]

2.2.3.3. Microscopic observations settings

Live cell images were taken at 37 °C in a live cell chambers of an Olympus FV1000 confocal microscope, with excitation at 440 nm for CFP, 488 nm for GFP, and 514 nm for YFP. Fixed cells were observed with a Zeiss LSM 510 Meta confocal microscope with an excitation of 458 nm for
CFP, 488 nm for GFP and Cy2, 514 nm for YFP, 543 nm for Cy3 and 633 nm for Cy5.
3. Results

3.1. Trafficking of MHC class I molecules

In order to differentiate between the retention and retrieval models of class I localization, I decided to determine the intracellular distribution of class I molecules in professional (lymphocytes) and non-professional antigen presenting cells (fibroblasts) by confocal immunofluorescence microscopy. No specific reagents exist that can detect peptide-receptive class I molecules by microscopy, since the conformation-specific antibodies that recognize different forms of class I molecules (peptide-bound and peptide-free) can only be used under native conditions. Therefore, in order to specifically detect peptide-receptive class I molecules, I have used mutant cell lines that are deficient in TAP function. The TAP transporter delivers peptides (which are generated by proteasomal degradation) to the lumen of the endoplasmic reticulum, and therefore in cells that lack TAP function, peptides are not transported into the ER, and class I molecules in the ER cannot be loaded with cytosolic peptides. It is not clear to what extent class I molecules in TAP-deficient cells have peptides bound to them. Some reports claim that they contain low-affinity peptides (De Silva et al., 1999; Townsend et al., 1989a), while others state that they contain no peptides at all (Williams et al., 1985). Theoretically, some peptides may be generated in the ER, for example from signal peptides, and these could be presented independently of TAP; indeed, HLA-A2 binds such peptides and takes them to the surface to a small extent in TAP-deficient cells (Wei and Cresswell, 1992). Alternatively, cytosolic peptides may be transported to the ER by means other than TAP. These pathways, if they exist, are likely to be much less prominent than TAP transport,
and in TAP-deficient cells, most class I molecules bind peptide that is added when the cells are lysed with detergent, indicating that if peptides are bound to them, these peptides are of a low affinity and dissociate easily (Townsend et al., 1989a). Thus, for the purpose of this thesis, the state of class I molecules in TAP-deficient cells will be termed 'peptide-receptive'.

3.1.1. Trafficking of MHC class I molecules in lymphocytes

To investigate the transport of peptide-receptive class I molecules in lymphocytes, I have used TAP-deficient T2 cells, which are derived from T1, a TAP proficient cell line. The T1 cell line was created as a fusion of the B lymphoblastoid cell line LCL 721.174 (TAP2 deletion) and the T cell line CEMR.3. T1 cells should therefore express LCL 721.174 (A2, B5) and CEMR encoded antigens (A1, Aw30, B8, B60), but one copy of chromosome six of CEMR.3 origin was spontaneously lost from the hybrid, and for this reason T1 cells are A1 and B8 negative. The T1 cell line expresses the HLA-A2 (A*0201), B5 (B*5101), B60, and Aw30 alleles of class I, and since this cell line contains functional TAP from CEMR, MHC class I proceeds normally to the cell surface. The T2 cell line was selected from T1. T2 cells have lost both CEMR-derived copies of the chromosome six, they express only the LCL 721.174-derived class I molecules (A2 and B5), and they do not have functional TAP transporter, which results in the retention of MHC class I molecules inside the cell (Salter et al., 1985; Salter and Cresswell, 1986).

In order to determine the steady state distribution of MHC class I molecules in T1 and T2 cells, I have used the monoclonal antibody HC10, which recognizes denatured HLA-B and C heavy chains and also some HLA-A
alleles (HLA-A3, A10, A28, A29, A30, A31 and A33; Grosse-Wilde, unpublished observations; Schnabl et al., 1990; Stam et al., 1990). With the HC10 antibody, I was therefore able to detect B5 and Bw6 in T1 cells and B5 in T2 cells.

In order to determine the intracellular distribution of MHC class I molecules, I also used organelle markers for the ER (calnexin (Hochstenbach et al., 1992) or the protein disulfide isomerase PDI (Macer and Koch, 1988)), ER exit sites (Sec31, Tang et al., 2000), the ERGIC (ERGIC-53 or p58, Lahtinen et al., 1996), the cis- (p23, Rojo et al., 2000) and trans-Golgi (GalT, Bretz et al., 1980), and lysosomes (CD63, Escola et al., 1998; Kobayashi et al., 2000; Piper and Luzio, 2001). Each colocalization experiment was repeated at least three times, and I observed the distribution of endogenous class I molecules in most of the cells present in each sample. To determine whether class I localizes in the given organelle, I always focused the microscope on the organelle marker first and then looked for the MHC class I distribution. For this reason, the stains for class I may be different between different panels of the same figures. In all figures, I have shown a single cell with such a distribution of class I as was characteristic for the majority of the cells.

In T1 cells, the steady-state level of endogenous class I is much higher than in T2 cells. This may be because these molecules are less stable in TAP-deficient cells, i.e., since they fail to associate with peptide, they may be degraded either in the ER or after endocytosis from the cell surface. While both T1 and T2 contain HLA-B5, T1 cells also express a Bw6 allele from CEM8.3, which was lost during the selection for T2 (see above). The expression of two
B alleles may be the reason why the total amount of class I detected in T1 is higher than in T2 cells.

In T1 wild type cells, class I molecules are present predominantly at the cell surface, and they also accumulate inside the cell (Figure 4, left panel). This intracellular accumulation localizes close to the Sec31 and ERGIC-53 positive compartments, but the best colocalization I obtained for p23, a marker that is specific for the cis side of the Golgi apparatus (Majoul et al., 2001). There is also partial colocalization with GalT, a trans-Golgi marker. There is no colocalization with CD63 (a lysosome marker); thus, those class I molecules that are retained intracellularly are not degraded in lysosomes.

Biochemically, the localization of class I molecules to the trans-Golgi and the cell surface of T1 cells was confirmed by endoglycosidase H (EndoH) digestion. EndoH removes only the sugar moieties of glycoproteins that have not been modified by the medial Golgi enzyme mannosidase II, (Tarentino and Maley, 1974; Tarentino et al., 1974). Therefore, resistance to EndoH digestion shows that a glycoprotein has reached the medial Golgi. In our and other laboratories, EndoH digestion of unlabeled lysates (B. Borchert) and of [35S] radiolabeled lysates following pulse-chase analysis (M. Al-Balushi) have demonstrated that in T1 cells, more than 50% of class I are EndoH resistant (Garstka et al., 2007).

In T2 cells, endogenous class I molecules are retained inside the cell, mostly in the ER, as confirmed by colocalization with calnexin (an ER membrane protein). In addition, they also accumulate in structures adjacent to ER exit sites and to the ERGIC. However, the best colocalization was obtained
with p23 (a cis-Golgi marker, Figure 4, right panel). In contrast to the situation in T1 cells, class I in T2 cells does not colocalize with GalT (trans-Golgi), and it does not become EndoH resistant (Al-Balushi and Borchert, Garstka et al., 2007). Like in T1 cells, endogenous class I in T2 cells does not colocalize with the lysosome marker, suggesting that peptide-receptive forms are not targeted for degradation.

The steady-state distribution of MHC class I molecules in T2 cells thus suggests that peptide-receptive forms leave the endoplasmic reticulum and become transported to the Golgi apparatus. Remarkably, in T1 cells, there is a similar intracellular accumulation of class I, and without being able to assess their peptide occupancy, I suggest that these may, like in T2 cells, represent peptide-receptive forms that proceed only as far as the cis-Golgi before being returned to the ER.

The accumulation of class I molecules in the cis-Golgi may be static, or it may represent, in a continuous cycling process between the ER and the Golgi apparatus, the station with the slowest rate of exit. To distinguish between these possibilities, one can apply a trafficking block out of the ERGIC by incubating the cells at 15 °C (Lippincott-Schwartz et al., 1990). Under these conditions, cycling molecules should become accumulated in the ERGIC while static populations should not shift. Upon 15 °C incubation of T2 cells, the intracellular pool of class I molecules (which in T1 and T2 cells at steady state best colocalized with the cis-Golgi marker) became redistributed to the ERGIC, and it colocalized with ERGIC-53 and also partially with Sec31, in a distribution characteristic for the ERGIC (Appenzeller-Herzog and Hauri,
2006), but not with p23 nor with GalT. Also, there was no colocalization of empty class I molecules with CD63 (Figure 5).

When the 15 °C block was released, class I molecules redistributed to the cis-Golgi (not shown). In T2 cells, colocalization with the cis-Golgi was maintained during a 20°C incubation, which blocks the exit from the Golgi apparatus (Matlin and Simons, 1983; Figure 6, right panel), with some fraction of molecules also in ERGIC. In T1 cells, the 20°C block resulted in the accumulation of class I molecules in the ERGIC and the cis-Golgi (probably a recycling pool of class I molecules) and in the trans-Golgi (possibly class I forms blocked at their exit from the Golgi apparatus to the cell surface).

Taken together, these data suggest that in lymphocytes, peptide-receptive forms of class I molecules can leave the endoplasmic reticulum and become transported through the ERGIC to the cis-Golgi, from where they are retrieved back to the ER. This supports the retrieval hypothesis of class I localization.

3.1.2. Trafficking of MHC class I molecules in fibroblasts

As the next step, I decided to determine the localization of different forms of class I molecules in non-professional antigen presenting cells (fibroblasts) and to compare it with lymphocytes. To approach this question, the fusion of the murine allele of class I, H-2D\textsuperscript{b}, with green fluorescent protein was used. This construct was then expressed in wild type (wt) and in TAP2-deficient (TAP2d) Chinese Hamster Ovary (CHO) cells to assess the colocalization with different organelle markers.
To ensure that the fusion of green fluorescent protein to class I molecules will not affect their trafficking, many different cells were observed and the level of the class I expression to its distribution were compared. An H-2D$^b$ fusion with the fluorescent protein, DsRed, (which is known to oligomerize) was constructed and its distribution in the cell was compared to H-2D$^b$-GFP. D$^b$ fused to GFP was uniformly distributed in the ER with only small intracellular accumulation; in contrast, D$^b$-DsRed molecules were localized to large clusters.

As for lymphocytes, I repeated each colocalization experiment at least three times, and I observed the distribution of H-2D$^b$ molecules in most of the cells in each sample. As the result, I have again shown representative single cells.

There is no difference in the expression level of H-2D$^b$ between wild type and TAP-deficient cells. This could be due to the fact that H-2D$^b$ is overexpressed in both cell lines.

In TAP2d CHO cells, class I localizes intracellularly. It mostly accumulates with the ER marker, calnexin (Figure 7, right panel). In addition, there is very good colocalization with Sec22 (ER plus ER exit sites), but not with Sec31 (ER exit sites only). Sec22 cycles between ER and cis-Golgi and is predominantly localized in the ER and the ERGIC (Hay et al., 1998). Good colocalization with this protein already suggests trafficking of class I out of the ER. In addition to the uniform distribution of empty class I molecules in the ER, there is also a small population of molecules that accumulates intracellularly, as for T2 cells. This population colocalizes well with p23, and it
Results

Malgorzata Garstka

is in close proximity to the Sec31 and p58 positive compartments. There is no colocalization with GalT, which shows that in TAP2d CHO cells, class I molecules do not reach the trans-Golgi. This was also confirmed by EndoH treatment of lysates (Al-Balushi and Borchert, Garstka et al., 2007). Peptide-receptive class I molecules also do not appear to be targeted for lysosomal degradation, since there is no colocalization with the lysosome marker, CD63. The steady state distribution suggests that empty class I molecules can be found in the cis-Golgi, and this was also confirmed by a 20°C block (Figure 9, right panel). However, in TAP2d cells at 20°C, H-2Dk-GFP can also be found in the trans-Golgi, which may be due to inefficient retention of these molecules (Ljunggren et al., 1990). When a 15°C block was applied, peptide-receptive class I relocalized to the ERGIC and also colocalized partially with Sec31, in a distribution characteristic for the ERGIC (Figure 8, right panel). The data suggest that in TAP2d CHO cells, like in TAP-deficient lymphocytes, class I molecules exit the ER and are transported to the cis side of Golgi apparatus, from where they are retrieved back to the endoplasmic reticulum.

In wild type fibroblasts, class I colocalizes predominantly with the ER markers (very good colocalization with calnexin and fairly good colocalization with Sec22, Figure 7, left panel). Some additional material colocalized with ER exit sites, the ERGIC, cis- and trans-Golgi, but not with lysosomes. In contrast to wild type lymphocytes, their cell surface expression was very low. Biochemical experiments in the laboratory have demonstrated that only about
10% of $D^b$ molecules in wt CHO cells are EndoH resistant (Al-Balushi and Borchert, not shown).

Why is the majority of mouse class I molecules in wild type CHO or Vero fibroblasts unable to proceed to the cell surface, and only a small population becomes EndoH resistant? I reasoned that this might be because lymphocytes are professional antigen presenting cells and fibroblasts are not. In fibroblasts, expression of the genes in the MHC is downregulated in comparison to the lymphocytes, TAP and tapasin proteins are often at very low levels, and therefore the amount of the components of the loading complex may be insufficient to load peptides onto the overproduced class I molecules. However, hamster class I molecules overexpressed in the CHO cells are exported to the cell surface (Tourdot et al., 2005); this suggests that $D^b$-GFP, expressed in hamster cells, does not interact efficiently with the hamster loading complex, and that therefore it can not bind optimal peptides which are required for its exit to the cell surface.

### 3.2. Peptide effect on the class I localization

The low amount of murine class I, which I observed at the cell surface of wild type CHO and Vero fibroblasts could be due to inefficient optimization of their peptide cargo in the ER. To investigate this hypothesis, I developed with the help of Irina Majoul (Royal Holloway University, London, UK) an assay that forces the supply of high-affinity peptide inside the cell. This approach was first used for H-2K$^b$-GFP molecules expressed in Vero cells and for the high affinity peptide, SIINFEKL (Falk et al., 1991), either unmodified or conjugated with the
fluorescent dye, Cy3, on the lysine side chain. Vero cells were electroporated with a plasmid that encodes H-2K\(^b\)-GFP and with SIINFEKL peptide, and they were observed after 24 hours. Strikingly, the direct supply of high affinity peptide to the cell led to the export of class I molecules to the cell surface. This was also the case for CHO cells and for a stable Vero H-2K\(^b\)-GFP cell line with microinjected peptide (not shown). The peptide effect is also seen in β\(_2\)m-deficient fibroblasts (Figure 10). This suggests that whatever the cause of intracellular retention of murine class I molecules in CHO or Vero fibroblasts, it can be overcome with high-affinity peptide, and that a similar effect leads to the egress of free heavy chains bound to peptide in β\(_2\)m-deficient cells.

3.2.1. Peptide can access the ER only upon electroporation

The effect of H-2K\(^b\)-GFP relocation from the ER to the cell surface upon addition of high affinity SIINFEKL peptide was visible only when peptide was electroporated or microinjected, but not when peptide (at a concentration of 300 nM or 1 µM) was added to the culture medium of the Vero/H-2K\(^b\)-GFP or β\(_2\)m-deficient fibroblasts/H-2K\(^b\)-GFP stable transfectants. The same results were obtained for stably transfected Vero H-2D\(^b\)-GFP cells and FAPGNYPAL peptide (not shown). Likewise, when peptide was not electroporated directly to the cells but added 4 hours after transfection, the effect was much weaker, and only low amounts of class I molecules were present at the cell surface 24 hours after transfection (not shown).

These data suggest that SIINFEKL and FAPGNYPAL peptides can enter the cell only upon electroporation (especially that they do not permeate,
under my experimental conditions, the plasma membranes of CHO and Vero cells), and that they are not transported to the ER by endocytosis and retrograde transport through the secretory pathway.

3.2.2. Peptide is electroporated to the cytosol and then transported to the ER by the TAP transporter

Another interesting question is whether peptide is electroporated directly into the endoplasmic reticulum, or whether it has access only to the cytosol and is then moved, from there, to the lumen of the ER by the TAP transporter.

To address this question, I applied the peptide electroporation assay to cells that are deficient in TAP function. TAP2d CHO cells were electroporated with FAPGNYPAL peptide and a plasmid encoding H-2D\(^b\)-GFP and observed after 24 hours. In these experiments, I determined the presence of D\(^b\)-GFP allele of class I at the cell surface by fluorescence microscopy. This approach gave information not only about the cell surface expression of H-2D\(^b\)-GFP, but also about the distribution of the class I molecules inside the cell. The quantification was done by scoring each cell either positive or negative for surface expression, and calculating the percentage of positive cells for the total (which was at least 200 cells in every case). To give more quantitative data, these experiments should be repeated and analyzed by flow cytometry.

There was no significant increase of H-2D\(^b\)-GFP cell surface expression in TAP2d cells, nor the difference in its distribution inside the cell, while in wild type cells, addition of the peptide caused an increase from 20.0 ±7.3 to 60.0 ±12.3 in the cell surface expression of H-2D\(^b\)-GFP (Figure 11).
suggests that the peptide is electroporated to the cytosol, not into the ER, and then transported into the ER in a TAP-dependent manner.

3.2.3. Peptide relocates class I from the ER to the Golgi apparatus and the cell surface

Electroporation of high-affinity peptide caused the relocation of H-2D\textsuperscript{b}-GFP and H-2K\textsuperscript{b}-GFP molecules to the cell surface within 24 hours. To follow the progress of the class I molecules through the intracellular compartments, I decided to observe the distribution of H-2D\textsuperscript{b}-GFP in Vero cells at different time points after the electroporation of FAPGNYPAL.

There are different stages in the export of H-2D\textsuperscript{b}-GFP to the cell surface. Within the first 12 hours after peptide electroporation, class I is present in the ER. At 14 hours, class I begins to accumulate at Golgi apparatus, and at 18 h, the first H-2D\textsuperscript{b}-GFP molecules could be seen at the cell surface. Within the next few hours, class I leaves the Golgi apparatus and occupies the cell surface until its complete saturation, which is observed 24 hours after transfection (Figure 12). Interestingly, prior to exit from the ER, an emphasis on the nuclear envelope is often seen (the 14 h time point in Fig. 12).

3.2.4. The carboxy terminus of the peptide is required for the export of peptide-class I complex to the cell surface

The length and the amino acid sequence are important features for tight binding of peptide to class I, i.e. for a low equilibrium K\textsubscript{D} and for slow dissociation of the peptide from the class I molecule (Falk et al., 1991; Fremont et al., 1992; Rammensee et al., 1995; Van Bleek et al., 1991; Young
et al., 1994; Zhang et al., 1992; Springer et al., 1998). However, it is not known which features of the peptide are necessary and sufficient to induce the export of the class I-peptide complex to the cell surface, and which protein assesses successful peptide binding to class I.

Optimal peptides of 8-10 amino acids with both anchor residues and both termini can move class I to the cell surface. What then about sub-optimal peptides that lack, for example, one anchor residue? To address this question, I used the peptide electroporation assay to investigate the ability of different peptides to move class I to the cell surface. Vero cells were electroporated with H-2D\textsuperscript{b}-GFP and different peptides at 100 and 300 nM concentrations. The FAPGNYPAL (FL9) peptide is a nonamer with asparagine and leucine as anchor residues. The peptides derived from FAPGNYPAL were: FAPGNYPAA (FA9), FAPGNYPA (FA8), FAPGNYP (FP7), FAPGNY (FY6), FAPGN (FN5), and NYPAL (NL5). As negative control, samples without peptide or with an irrelevant peptide (ILKEPVGHV, IV9) were used (Figure 13).

Optimal peptides, which possess both anchor residues and both termini, stabilize class I at the cell surface in 51.4 ±7.3 (FAPGNYPAL, 100 nM) and in 55.8 ±8.4% (FAPGNYPAL, 300 nM) of cells. When the side chain of the C terminal anchor residue, leucine, which binds into the F pocket of the class I peptide binding site, is substituted by alanine, there is a reduction in cell surface expression to 35.3 ±4.0% (FAPGNYPAA, 100 nM) and 47.7 ±7.5% (FAPGNYPAA, 300 nM). When the C-terminal amino acid of the peptide is lacking completely (which removes both the interaction of the anchor residue
side chain with the F pocket and the interaction of the terminal carboxylate group with both lateral helices of the binding groove), there is a further decrease to 29.3 ±8.3% (FAPGNYPA, 100 nM) and 34.1 ±6.0% (FAPGNYPA, 300 nM). C-terminally truncated peptides that lack two or more residues do not move class I to the cell surface to a higher degree than it is seen for sample without peptide (FAPGNYP, FAPGNY, FAPGN). In contrast, the five-residue peptide (NYPAL) that lacks four amino acids from the amino terminus of the optimal peptide is able to move class I to the cell surface in up to 36.9 ±3.8% of cells (Figure 13).

According to tryptophan fluorescence denaturation experiments of recombinant H-2D\textsuperscript{b} refolded with the different peptides, both NYPAL and FAPGNYP bind to H-2D\textsuperscript{b} (with FAPGNYP binding somewhat more tightly) (Tigan and Springer, not shown). However, only NYPAL can move class I to the cell surface, while FAPGNYP shows an effect similar to the negative control. One possible reason for this lack of effect of FAPGNYP is that it may not be transported by TAP to the ER and therefore cannot bind to class I. First, peptides with a proline at position three do not bind well to the TAP transporter (Neisig et al., 1995), and second, peptides shorter than eight amino acids are not a good substrate for TAP (van Endert et al., 1994). To investigate this possibility, I tested the ability of FAPGNYP to compete with other peptides for TAP transport and/or class I binding, i.e., to suppress the ability of other peptides to induce cell surface transport of class I. Vero cells were electroporated with H-2D\textsuperscript{b}-GFP and a combination of peptides at 100 nM concentration each. Two combinations, FAPGNYP plus NYPAL, and
FAPGNYP plus FAPGNYPAL, were investigated. As the negative control, samples without peptide, with irrelevant peptide (ILKEPVGHV), or with FAPGNYP peptide were used. As positive controls, samples with NYPAL or FAPGNYPAL peptides were used.

There is a reduction from 40.5 ±10.2% to 28.5 ±3.2% in the cell surface expression of H-2D<sup>b</sup>-GFP when FAPGNYP+NYPAL were used instead of NYPAL only. For FAPGNYPAL, there is a decrease from 64.5 ±8.8 to 29.5 ±7.4% (Figure 14). These data suggest that FAPGNYP competes with NYPAL or FAPGNYPAL for transport into the ER, or for binding to H-2D<sup>b</sup>-GFP. To determine at which stage this competition takes place, I have investigated the combination of FAPGNYPAL+ILKEPVGHV, both at the 100 nM concentration. If the competition is at the level of TAP transporter, I would see the decrease in the cell surface expression of D<sup>b</sup>-GFP molecules, because FAPGNYPAL is much worse substrate for TAP than ILKEPVGHV due to proline at position three in its sequence.

There is no difference in the cell surface expression of H-2D<sup>b</sup>-GFP when FAPGNYPAL+ILKEPVGHV were used instead of FAPGNYPAL only (66.0 ±7.11% and 64.5 ±12.5%, respectively, Figure 14). This demonstrates that peptides at the concentration 100 nM do not compete for binding to TAP (although in this experiment, I did not actually demonstrate that ILKEPVGHV is transported by TAP).

My data also show that FAPGNYP has access to the endoplasmic reticulum and that it competes with NYPAL and FAPGNYPAL for binding to class I. But even though it can bind to class I, FAPGNYP cannot move it to the
cell surface. This suggests that the two C-terminal residues in FAPGNYPAL peptide are more important in class I export to the cell surface than four N-terminal residues.

3.3. The effect of tapasin on class I localization

Taking together the results of the last sections, it seems that the optimal peptide allows for the export of class I to the cell surface, but that it is not required for class I exit from the ER and recycling from the cis-Golgi. This suggests that the control of class I surface transport takes place at a location other than the ER, probably either the ERGIC or the cis-Golgi. To begin to elucidate how trafficking of class I is controlled, I decided to investigate the role of the components of the loading complex on class I distribution following peptide electroporation.

The most important protein for peptide editing appears to be tapasin. In wild type cells, class I molecules bind peptides of increasing affinity until they exit to the cell surface, but in cells that lack tapasin, most class I molecules (the 'tapasin-dependent' ones, for example HLA-B*4402 or H-2D^b), cannot bind high affinity peptides and do not traffic to cell surface. They do not reach the medial Golgi since they remain sensitive to EndoH digestion (Williams et al., 2000).

It is not known how tapasin brings about high-affinity peptide binding to class I (for some models see Wright et al., 2004). Clearly, since tapasin helps class I to bind high-affinity peptide, it supports its transport to the cell surface. On the other hand, since tapasin binds to empty class I molecules, it may also retain them in the ER or retrieve them from the cis-Golgi due to its C-terminal double lysine (KKXX) motif (Ortmann et al., 1997, Paulson et al., 2002).
In order to determine the influence of tapasin on the trafficking of class I, I expressed the H-2D\textsuperscript{b}-GFP construct in mouse ear fibroblasts (MEF) both from wild type (wt) and from tapasin knock-out mice (tpn\textsuperscript{-/-}) (Grandea et al., 2000). I repeated this experiment three times. H-2D\textsuperscript{b}-GFP molecules that are overexpressed in wild type MEF bind endogenous peptides and are transported to the cell surface in 64 ±2.9% of the cells (Figure 15). In tapasin-deficient MEF, H-2D\textsuperscript{b}-GFP is present at the cell surface only in 18.2 ±1.6% of cells.

I then used the peptide electroporation assay in tapasin\textsuperscript{-/-} MEF to determine whether the optimal peptide (FAPGNYPAL) can move H-2D\textsuperscript{b}-GFP molecules to the cell surface in tapasin\textsuperscript{-/-} cells when it is present in large excess (300 nM). As controls, wild type MEF with and without FAPGNYPAL peptide were used.

For wild type MEF, I found no difference between the samples with and without peptide. In both samples, there was cell surface expression of D\textsuperscript{b}-GFP 24 hours after transfection seen in majority of cells (64.0 ±2.9% and 64.5 ±3.9% respectively). In tapasin-negative cells, there is only 18.2 ±1.6% of cells with cell surface expression of class I without extra peptide. Upon the addition of the FAPGNYPAL peptide, there is an increase in the cell surface expression of H-2D\textsuperscript{b}-GFP in tapasin\textsuperscript{-/-} MEF from 18.2 ±1.6 to 39.7 ±2.3 %, but cell surface expression is not as strong as for wild type cells. The distribution of H–2D\textsuperscript{b} in tapasin-deficient cells upon electroporation of peptide is different from that in wild type cells (Figure 15a): they are localized around the nucleus with only a subtle cell surface stain. Considering the transport pathway of class I molecules to the cell surface (ER - nuclear envelope - Golgi - surface), one would assume that there is a movement of H-2D\textsuperscript{b}-GFP molecules to the cell surface, however
24 hours after transfection, not all of them have reached the cell surface. Perhaps in tpn\(^{+}\) MEF, the loading of transfected peptide is slower, or less efficient, than in the wild type cells.

When H-2D\(^{b}\)-GFP is transiently transfected into MEF, the molecules appear at the cell surface (in contrast to the same molecules in Vero or CHO cells, see above). In Vero cells, FAPGNYP partially inhibited the D\(^{b}\) that was stimulated to exit the ER by electroporation of FAPGNYPAL. I therefore next wanted to find out whether in wild type MEF, where presumably the interaction of D\(^{b}\) with the loading complex is functional, electroporated FAPGNYP peptide would have the same effect and prevent the exit of D\(^{b}\)-GFP. I therefore electroporated wild type MEF with H-2D\(^{b}\)-GFP and 300 nM of FAPGNYP peptide. As the control, a sample without peptide was used.

There is a reduction (from 62.7 ±1.0 to 50.0 ±4.9\%) in the cell surface expression of H-2D\(^{b}\)-GFP when FAPGNYP was used compared to the no peptide control (Figure 16b). This is accompanied by a difference in the distribution of H-2D\(^{b}\)-GFP in those cells electroporated with FAPGNYP peptide. Class I is mostly accumulated around the nucleus with not as strong cell surface stain as for cells without FAPGNYP peptide (Figure 16a). These data need to be confirmed by flow cytometry to give a more precise quantification.

### 3.4. The effect of calreticulin on class I localization

Calreticulin is a lectin that is important for the proper folding of glycoproteins, including class I molecules. It has also been reported to bind misfolded proteins independent of their glycosylation (Saito et al., 1999). Calreticulin binds to the empty class \(-\beta_{2}m\) dimer prior to peptide binding and is a
component of the loading complex (Gao et al., 2002). Since calreticulin binds to empty class I molecules, it may localization in the cell. To determine the role of calreticulin in the transport of class I, I expressed the H-2D<sup>b</sup>-GFP construct in wild type (K41) and calreticulin-deficient (K42) mouse fibroblasts (kindly supplied by T. Elliott). H-2D<sup>b</sup>-GFP molecules that are overexpressed in wild type K41 cells bind endogenous peptides and are transported to the cell surface in 66 ±0.7% of the cells. In calreticulin-deficient cells, H-2D<sup>b</sup>-GFP is transported to the cell surface only in 16.0 ±0.7% of cells (Figure 17a). In addition, I could see a punctate distribution of class I in these cells, which suggests that these molecules are targeted for degradation in lysosomes.

I next used the peptide electroporation assay with K42 fibroblasts to determine whether the optimal peptide (FAPGNYPAL) can move H-2D<sup>b</sup>-GFP molecules to the cell surface in these cells when it is present in large excess (300 nM). As a control, wild type cells with and without FAPGNYPAL peptide were used.

There is almost no difference for wild type cells when the optimal peptide was electroporated, and an increase (from 16.0 ±0.7% to 26.5 ±2.5%) for calreticulin-deficient cells. In addition, the distribution of class I with electroporated peptide is more uniform than for the sample without the peptide, but less dense compared to wild type cells (Figure 17). These data suggest that calreticulin is required for class I to efficiently bind endogenous high-affinity peptide, and that lack of calreticulin can be partially overcome by an excess of optimal peptide.
### Figure 4

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The distribution of endogenous class I molecules in wild type (T1, left panel) and TAP-deficient (T2, right panel) lymphocytes at 37°C. Confocal immunofluorescence microscopy with compartment markers: calnexin (ER), Sec22 (ER and exit sites), Sec31 (ER exit sites), ERGIC53 (ERGIC), p23 (cis-Golgi), GalT (trans-Golgi), CD63 (lysosomes). Representative single cells are shown. Scale bar 10 µm.
Figure 5

The distribution of endogenous class I molecules in wild type (T1, left panel) and TAP-deficient (T2, right panel) lymphocytes at 15°C. Confocal immunofluorescence microscopy with compartment markers: calnexin (ER), Sec22 (ER and exit sites), Sec31 (ER exit sites), ERGIC53 (ERGIC), p23 (cis-Golgi), GalT (trans-Golgi), CD63 (lysosomes). Representative single cells are shown. Scale bar 10 µm.
**Figure 6**

The distribution of endogenous class I molecules in wild type (T1, left panel) and TAP-deficient (T2, right panel) lymphocytes at 20°C. Confocal immunofluorescence microscopy with compartment markers: calnexin (ER), Sec22 (ER and exit sites), Sec31 (ER exit sites), ERGIC53 (ERGIC), p23 (cis-Golgi), GalT (trans-Golgi), CD63 (lysosomes). Representative single cells are shown. Scale bar 10 µm.
**Figure 7**

<table>
<thead>
<tr>
<th>Marker</th>
<th>CHO</th>
<th>TAP2d</th>
</tr>
</thead>
<tbody>
<tr>
<td>calnexin</td>
<td><img src="overlay1.png" alt="Overlay" /></td>
<td><img src="overlay2.png" alt="Overlay" /></td>
</tr>
<tr>
<td>Sec22</td>
<td><img src="overlay3.png" alt="Overlay" /></td>
<td><img src="overlay4.png" alt="Overlay" /></td>
</tr>
<tr>
<td>Sec31</td>
<td><img src="overlay5.png" alt="Overlay" /></td>
<td><img src="overlay6.png" alt="Overlay" /></td>
</tr>
<tr>
<td>p58</td>
<td><img src="overlay7.png" alt="Overlay" /></td>
<td><img src="overlay8.png" alt="Overlay" /></td>
</tr>
<tr>
<td>p23</td>
<td><img src="overlay9.png" alt="Overlay" /></td>
<td><img src="overlay10.png" alt="Overlay" /></td>
</tr>
<tr>
<td>GalT</td>
<td><img src="overlay11.png" alt="Overlay" /></td>
<td><img src="overlay12.png" alt="Overlay" /></td>
</tr>
<tr>
<td>CD63</td>
<td><img src="overlay13.png" alt="Overlay" /></td>
<td><img src="overlay14.png" alt="Overlay" /></td>
</tr>
</tbody>
</table>

The distribution of H-2D\(^b\) molecules in wild type (CHO, left panel) and TAP-deficient (TAP2d, right panel) fibroblasts at 37°C. Confocal immunofluorescence microscopy with compartment markers: calnexin (ER), Sec22 (ER and exit sites), Sec31 (ER exit sites), p58 (ERGIC), p23 (cis-Golgi), GalT (trans-Golgi), CD63 (lysosomes). Representative single cells are shown. Scale bar 10 µm.
Figure 8

The distribution of H-2D^b molecules in wild type (CHO, left panel) and TAP-deficient (TAP2d, right panel) fibroblasts at 15°C. Confocal immunofluorescence microscopy with compartment markers: calnexin (ER), Sec22 (ER and exit sites), Sec31 (ER exit sites), p58 (ERGIC), p23 (cis-Golgi), GalT (trans-Golgi), CD63 (lysosomes). Representative single cells are shown. Scale bar 10 µm.
Figure 9

The distribution of H-2D<sup>b</sup> molecules in wild type (CHO, left pannel) and TAP-deficient (TAP2d, right pannel) fibroblasts at 20°C. Confocal immunofluorescence microscopy with compartment markers: calnexin (ER), Sec22 (ER and exit sites), Sec31 (ER exit sites), p58 (ERGIC), p23 (cis-Golgi), GalT (trans-Golgi), CD63 (lysosomes). Representative single cells are shown. Scale bar 10 µm.
Figure 10

Vero  CHO  β₂m⁻ fibroblasts

H-2K<sup>b</sup>-GFP relocalizes from the ER to the cell surface of fibroblasts following electroporation with SIINFEKL peptide. Vero, CHO and β₂m⁻ fibroblasts were electroporated with a plasmid encoding H-2K<sup>b</sup>-GFP and SIINFEKL (SL8) peptide, incubated for 24 hours and visualized. Scale bar 10 µm (Exp. 20.5, 20.7, 20.9, 20.11, 21.37, 21.39, 22.2, 22.3, 22.5).
Figure 11A

<table>
<thead>
<tr>
<th>CHO wt</th>
<th>TAP2d</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1" alt="Image of CHO wt H-2D&lt;sup&gt;b&lt;/sup&gt;-GFP" /></td>
<td><img src="image2" alt="Image of TAP2d H-2D&lt;sup&gt;b&lt;/sup&gt;-GFP" /></td>
</tr>
<tr>
<td><img src="image3" alt="Image of CHO wt FL9" /></td>
<td><img src="image4" alt="Image of TAP2d FL9" /></td>
</tr>
</tbody>
</table>
The peptide delivery to the ER is TAP-dependent. Wild type (CHO) and TAP-deficient (TAP2d) cells were electroporated with a plasmid encoding H-2D\(^b\)-GFP and 300 nM of FAPGNYPAL (FL9) peptide, incubated for 48 hours and visualized. As a control cells without peptide were used.

a. Confocal immunofluorescence microscopy. Representative field of cells are show. Scale bar 10 \(\mu\)m.

b. The percentage of cells showing cell surface expression of H-2D\(^b\)-GFP was determined for each sample. The results are mean value from free experiments (Exp. 21.38, 21.39, 21.42).
Figure 12

Time course of the H-2D\textsuperscript{b}-GFP-FAPGNYPAL transport to the cell surface. Vero cells were electroporated with a plasmid encoding H-2D\textsuperscript{b}-GFP and 300 nM of FAPGNYPAL peptide, incubated for 24 hours and visualized. As a negative control cells without peptide were used. Confocal immunofluorescence microscopy. Panels show single cells representative for the most common localizations of H-2D\textsuperscript{b}-GFP at different times. Scale bar 10 µm (Exp. 24.14, 24.25, 24.26, 24.31, 24.35).
Figure 13A
Figure 13B

<table>
<thead>
<tr>
<th>Peptide</th>
<th>% of cell surface</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>100 nM</td>
</tr>
<tr>
<td>nc</td>
<td>16.8±2.1</td>
</tr>
<tr>
<td>ILKEPVGHV (IV9)</td>
<td>20.5±3.2</td>
</tr>
<tr>
<td>FAPGNY (FY6)</td>
<td>15.0±0.0</td>
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<tr>
<td>FAPGNY (FY6)</td>
<td>8.5±0.0</td>
</tr>
<tr>
<td>FAPGNYP (FP7)</td>
<td>13.0±1.3</td>
</tr>
<tr>
<td>FAPGNYP (FA8)</td>
<td>29.3±8.3</td>
</tr>
<tr>
<td>FAPGNYPAA (FA9)</td>
<td>35.3±4.0</td>
</tr>
<tr>
<td>FAPGNYPAL (FL9)</td>
<td>51.4±7.3</td>
</tr>
<tr>
<td>NYPAL</td>
<td>32.9±5.3</td>
</tr>
</tbody>
</table>

Not all peptides induce the transport of class I to the cell surface. Vero cells were electroporated with a plasmid encoding H-2D\(^{b}\)-GFP and 100 nM or 300 nM of different peptides, incubated for 24 hours and visualized. As a negative control cells without peptide were used.

a. Confocal immunofluorescence microscopy. Representative single cells are shown. Scale bar 10 μm

a. The percentage of cells showing cell surface expression of H-2D\(^{b}\)-GFP was determined for each sample. Results are mean value from seven experiments (Exp.24.7-24.9, 24.16, 24.24, 24.34, 24.36).
Figure 14A
FAPGNYP can not move class I to the cell surface, but it competes with NYPAL for binding to class I. Vero cells were electroporated with a plasmid encoding H-2D<sup>b</sup>-GFP and 100 nM or 300 nM of different peptides, incubated for 24 hours and visualized. As a negative control cells without peptide were used.

a. Confocal immunofluorescence microscopy. Representative single cells are shown. Scale bar 10 µm.

a. The percentage of cells showing cell surface expression of H-2D<sup>b</sup>-GFP was determined for each sample. Results are mean value from three experiments (Exp.24.34, 24.36, 24.38).
Figure 15A

<table>
<thead>
<tr>
<th>MEF wt</th>
<th>MEF tapasin&lt;sup&gt;-/-&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
</tr>
<tr>
<td><img src="image3.png" alt="Image" /></td>
<td><img src="image4.png" alt="Image" /></td>
</tr>
</tbody>
</table>
**Figure 15B**

<table>
<thead>
<tr>
<th>Sample</th>
<th>% of cell surface</th>
</tr>
</thead>
<tbody>
<tr>
<td>MEF wt, H-2D&lt;sup&gt;b&lt;/sup&gt;-GFP</td>
<td>64.0±2.9</td>
</tr>
<tr>
<td>MEF wt, H-2D&lt;sup&gt;b&lt;/sup&gt;-GFP, FL9</td>
<td>64.5±3.9</td>
</tr>
<tr>
<td>MEF tapasin&lt;sup&gt;-/-&lt;/sup&gt;, H-2D&lt;sup&gt;b&lt;/sup&gt;-GFP</td>
<td>18.2±1.6</td>
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<tr>
<td>MEF tapasin&lt;sup&gt;-/-&lt;/sup&gt;, H-2D&lt;sup&gt;b&lt;/sup&gt;-GFP, FL9</td>
<td>39.7±2.3</td>
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</table>

The tapasin effect on the distribution of class I. Wild type (MEF wt) and tapasin-deficient (MEF TP<sup>-/-</sup>) cells were electroporated with a plasmid encoding H-2D<sup>b</sup>-GFP and 300 nM of FAPGNYPAL (FL9) peptide, incubated for 24 hours and visualized. As a negative control cells without peptide were used.

- **a.** Confocal immunofluorescence microscopy. Representative single cells are shown. Scale bar 10 µm.
- **a.** The percentage of cells showing cell surface expression of H-2D<sup>b</sup>-GFP was determined for each sample. The results are mean value from three experiments (Exp. 36.7, 36.22, 36.24).
Figure 16

A

![H-2D\textsuperscript{b}-GFP](image1)

B

<table>
<thead>
<tr>
<th>Peptide</th>
<th>% of cell surface</th>
</tr>
</thead>
<tbody>
<tr>
<td>MEF wt</td>
<td>62.7±1.0</td>
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<tr>
<td>MEF wt, FP7</td>
<td>50.0±4.9</td>
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</tbody>
</table>

FAPGNYP competes with endogenous peptides for binding to class I. MEF wt were electroporated with a plasmid encoding H-2D\textsuperscript{b}-GFP and 300 nM of FAPGNYP (FP7) peptide, incubated for 24 hours and visualized. As a positive control cells without peptide were used.

a. Confocal immunofluorescence microscopy. Representative populations of cells are shown. Scale bar 10 μm.

a. The percentage of cells showing cell surface expression of H-2D\textsuperscript{b}-GFP was determined for each sample. Results are mean value from three experiments (Exp. 36.19, 36.21, 36.24).
Figure 17A

K41(wt)  K42 (calreticulin<sup>−/−</sup>)

![Image of fluorescence microscopy results showing H-2D<sup>b</sup>-GFP and FL9 signals for K41(wt) and K42 (calreticulin<sup>−/−</sup>)]
Figure 17B

<table>
<thead>
<tr>
<th>Sample</th>
<th>% cell surface</th>
</tr>
</thead>
<tbody>
<tr>
<td>K41, H-2D^b-GFP</td>
<td>66.0±0.7</td>
</tr>
<tr>
<td>K41 H-2D^b-GFP, FL9</td>
<td>67.5±4.6</td>
</tr>
<tr>
<td>K42, H-2D^b-GFP</td>
<td>16.0±0.7</td>
</tr>
<tr>
<td>K42 H-2D^b-GFP, FL9</td>
<td>26.5±2.5</td>
</tr>
</tbody>
</table>

The calreticulin effect on the distribution of class I. Wild type (K41) and calreticulin-deficient (K42) cells were electroporated with a plasmid encoding H-2D^b-GFP and 300 nM of FAPGNYPAL (FL9) peptide, incubated for 24 hours and visualized. As a negative control cells without peptide were used.

a. Confocal immunofluorescence microscopy. Representative single cells are shown. Scale bar 10 µm.

b. The percentage of cells showing cell surface expression of H-2D^b-GFP was determined for each sample. The results are mean value from two experiments (Exp. 37.1, 37.9).
4. Discussion

4.1. Trafficking of MHC class I molecules

In the experiments described above, I have investigated the trafficking of the class I molecules in lymphocytes by staining with the HC10 monoclonal antibody and confocal fluorescence microscopy. HC10 recognizes free heavy chains and denatured HLA-B, C, and some A alleles. Even though HC10 recognizes only denatured class I molecules, I have assumed that the staining pattern represents the distribution of all three forms of class I (free heavy chain, peptide-receptive dimer, and trimeric complex, since through the fixation with paraformaldehyde, the latter two forms have denatured and converted to the first. The peptide- and β₂m-specific antibody, W6/32, did not give a specific staining signal after the fixation procedure (data not shown).

I have shown that in TAP-deficient T2 lymphocytes, MHC class I molecules accumulate at the cis side of the Golgi apparatus at steady state. They do not become EndoH resistant, which indicates that they cannot proceed further in the secretory pathway. When at 15 °C vesicular traffic out of the ERGIC is blocked, the accumulation of class I in the cis-Golgi disappears, and instead an accumulation in the ERGIC is seen. Since the class I molecules that were previously in the cis-Golgi cannot have moved to the cell surface, they must have returned from the cis-Golgi to the ERGIC, either directly or via the ER. Thus, the results of the 15 °C block experiment suggest that peptide-receptive class I molecules continuously cycle between the ERGIC and the cis-Golgi. To find out whether this accumulation consists of free heavy chain or peptide-receptive
dimers, I have also investigated the distribution of mouse free heavy chains in β₂m-negative fibroblasts in respect to the organelle markers. I could see only ER stain for these molecules, but no intracellular accumulation (not shown). From these observations, together with the results obtained with HC10 antibody, I propose cycling through the cis-Golgi for peptide-receptive class I dimers. In accordance with my data, Britta Borchert and Mohammed Al-Balushi in our laboratory have shown that peptide-receptive MHC class I molecules are present in COPII (ER-to-ERGIC) transport vesicles in T2 cells (Garstka et al., 2007). Taken together, these data support the recycling hypothesis, namely that peptide-receptive class I molecules exit the Endoplasmic Reticulum in COPII vesicles and are transported to the ERGIC, and then to the cis-Golgi. From the cis-Golgi, they are retrieved back to the ER (perhaps via the ERGIC, Figure 18). Interestingly, a similar (but smaller) pool of class I molecules can be found accumulated in the cis-Golgi in wild type T1 lymphocytes; this suggests that in wild type lymphocytes, a similar recycling mechanism may operate for peptide-receptive class I molecules. This is again supported by Mohammed Al-Balushi's detection of peptide-receptive class I molecules in COPII vesicles from T1 cells.

Since many previous studies on MHC class I trafficking were performed in fibroblasts (Baas et al., 1992; Day et al., 1995; Hsu et al., 1991; Paulsson et al., 2002; Petcheva et al., 2001; Spiliotis et al., 2000; Spiliotis et al., 2002), I have also investigated the distribution of class I molecules in fibroblasts using class I fusion proteins with green fluorescent protein to further test the recycling model. Such use of overexpressed GFP fusions in the study of protein trafficking can theoretically cause artifacts for two reasons:
Figure 18: Trafficking of MHC class I molecules. All forms of MHC class I can exit the ER and be transported to the ERGIC and cis-Golgi, but only forms bound with high affinity peptides can proceed further to the cell surface, while peptide-receptive class I molecules are retrieved back to the endoplasmic reticulum. (Figure courtesy of Patrycja Kozik and Sebastian Springer.)

- First, GFP tends to oligomerize (Jain et al., 2001; Shaner et al., 2005; Zacharias 2002), and this can influence protein distribution, for example by the retention of the GFP-protein fusion in the ER, while the unfused wild type protein is exported.
- Second, overexpression of a protein can produce artifacts, since it might cause the export of a protein that is not normally exported due to the saturation of the quality control machinery that cannot retain all these molecules any more (Hammond and Helenius, 1994; Hermosilla et al., 2004; Kincaid and Cooper, 2006; Pröp sting et al., 2003; Spear and Ng, 2003).
To be as certain as possible that the localization of the H-2D^b-GFP molecules is not an overexpression artifact, I observed many different cells with different levels of the class I expression. I compared the level of the class I expression to its distribution, and there was no difference between the level of the expression and the intracellular localization. In addition, I compared the distribution of D^b-GFP and D^b-DsRed constructs. DsRed molecules tend to oligomerize, and fusing DsRed to another protein will have an effect on this protein's localization. I have therefore compared D^b-DsRed molecules (which oligomerized and were predominantly localized in large clusters) to the expression of the D^b-GFP construct. The distribution of the H-2D^b-GFP construct was uniform (mostly in the ER), with only a small accumulation that did not resemble that of the D^b-DsRed clusters.

Once I was reasonably certain that the H-2D^b-GFP construct does not generate artifacts, I used it to investigate the distribution of these molecules in fibroblasts to confirm the recycling model of class I localization seen in lymphocytes, and to show that the trafficking of class I-GFP molecules in non-professional antigen presenting cells resembles the transport of endogenous molecules in APC.

In TAP-deficient fibroblasts, like in lymphocytes, class I molecules are accumulated at the cis side of the Golgi apparatus at steady state, and they relocalize to the ERGIC during a 15° C block. These results confirm the data obtained by the group of Klausner (Hsu et al., 1991) who investigated the intracellular trafficking of the mouse allele of class I, H-2K^d, in fibroblasts by sucrose fractionation, electron microscopy, and fluorescence microscopy.
these methods, they detected K\textsuperscript{d} molecules with the SF1-1.1.1 monoclonal antibody. Using the electron microscopy technique, they showed that H-2K\textsuperscript{d} molecules are present in the ER at 37°C, but redistribute to the Golgi apparatus upon the temperature shift to 16°C. By density gradient, they show that at 37°C, H-2K\textsuperscript{d} localizes mostly to the endoplasmic reticulum, since it is present in the fractions that are positive for the 90K protein (ER marker). However, at 16°C, class I is predominately found in the fractions that are positive for the \textit{trans}-Golgi marker, galactosyltransferase. The density gradient did not allow them to separate \textit{cis} from \textit{trans}-Golgi, and therefore these data seem to show that class I redistributes to the Golgi apparatus upon 16°C block. But, as for the electron microscopy, one cannot exclude that class I is present in the ERGIC and not in the \textit{cis}-Golgi. The authors confirmed and expanded these data by immunofluorescence microscopy, colocalizing class I with mannosidase II (\textit{trans}-Golgi marker) at 16°C, and at 37°C upon nocodazole treatment (which causes microtubule depolymerization), but not at the steady state. This experiment suggests that class I cycle through the ERGIC but not through the \textit{trans}-Golgi, and it leaves open the question whether H-2K\textsuperscript{d} molecules are retrieved to the ER from the ERGIC or from the \textit{cis}-Golgi. Using fluorescence microscopy, 15°C and 20°C blocks and different organelle markers, I have now shown that class I molecules cycle through the \textit{cis}-Golgi. I have shown that class I is mostly present in the endoplasmic reticulum at the steady state, but that in addition, it also accumulates intracellularly. I have specifically identified this accumulation to partially colocalize with p58 (ERGIC marker) but mostly with p23 (\textit{cis}-Golgi marker). Together with the biochemical data, which show that these molecules are
present in the COPII vesicles, but do not become EndoH resistant (Borchert, not shown), I could confirm the recycling model for peptide-receptive class I molecules in fibroblasts (Hsu et al., 1991, Baas et al., 1992, Day et al., 1995, Paulsson et al., 2002). In addition, I have shown the same recycling pattern for endogenous class I molecules in the lymphocytes, which suggests that there is no principal difference between the trafficking of peptide-receptive class I molecules in professional and nonprofessional antigen presenting cells, and that peptide editing and class I exit from the ER are independent events.

Surprisingly, however, I found that the trafficking of H-2D\textsuperscript{b}-GFP molecules in wild type fibroblasts does differ from the trafficking of endogenous class I molecules in wild type lymphocytes. In CHO cells, the mouse allele of class I, H-2D\textsuperscript{b}-GFP, localizes mostly to the ER with some accumulation at the cis-Golgi. However, there is no cell surface expression and only partial accumulation in the trans-Golgi. The localization of H-2D\textsuperscript{b}-GFP in fibroblasts is almost the same for the wild type and the TAP-deficient cells. There is also no cell surface expression of the other mouse allele, H-2K\textsuperscript{b}-GFP, in both CHO and Vero cells. This could be due to the species mismatch (with mouse class I molecules in hamster or monkey cells). Since hamster class I molecules that are fused with GFP do proceed to the cell surface in wild type CHO cells (Tourdot et al., 2005), I would assume that the generation of peptides and their transport into the ER are not generally defective in CHO or Vero cells. Therefore, the likely explanation why H-2D\textsuperscript{b}-GFP is not at the cell surface of CHO, and H-2K\textsuperscript{b}-GFP at the cell surface of CHO and Vero cells, is that the loading of mouse class I molecules with high-affinity peptides is inefficient in hamster or monkey
fibroblasts. This could be due to the inability of the D\(^b\)-GFP or K\(^b\)-GFP molecules to functionally interact with the loading complex in these cells and to optimize their ligand peptides. Another possible explanation of the intracellular localization of mouse class I molecules in hamster or monkey cells is their inefficient interaction with the endogenous \(\beta_2\)m and the retention of free heavy chains in the ER due to their interaction with calnexin. Indeed, it was shown that the cell surface expression of K\(^b\) allele of class I is strongly reduced in \(\beta_2\)m-negative cells, and that H-2K\(^b\) molecules are rapidly internalized and degraded. D\(^b\), in \(\beta_2\)m-negative cells, can still be exported to the cell surface, but the majority of the molecules are retained intracellularly and targeted for degradation (Allen et al., 1986; Machold et al., 1995, Williams et al., 1989). It was also shown that both H-2D\(^b\) and H-2D\(^b\) heavy chains require peptides to reach the cell surface of \(\beta_2\)m-negative cells (Machold et al., 1995). Therefore, I decided to supply a large excess of the D\(^b\)-GFP optimal peptide (FAPGNYPAL, FL9) to the hamster fibroblasts. I have used electroporation to allow the peptide to enter the cell, since incubation of the cells with the peptide did not cause its internalization. This is in contrast to the results by another group, which has shown that incubation of cells with peptide caused its access to the class I molecules in the secretory pathway (Day et al., 1995). This group has used RMA and RMA-S cells, both of which expose peptide-receptive forms of D\(^b\) and K\(^b\) alleles of class I molecules at the cell surface. Therefore, peptides were bound to the class I present at the cell surface, but also to the intracellular class I molecules. The presence of class I-peptide complexes at the cell surface in RMA-S cells would suggest that in these cells, peptide crosses the membrane in order to be delivered to the ER. I have shown for
fibroblasts, that the electroporation is required to deliver peptides inside the cell (to the cytosol) and then TAP transporter, to deliver peptides to the ER.

In my experiments, electroporation of the peptide moved H-2D\textsuperscript{b}-GFP molecules to the cell surface in a TAP-dependent manner. I obtained the same results for H-2K\textsuperscript{b}-GFP with SIINFEKL peptide in CHO and Vero cells. Thus, very large amounts of optimal peptide can overcome the loading deficiency of murine class I in hamster or monkey cells. In murine lymphocytes, in contrast, where the loading of high-affinity peptides is more efficient, most of the murine class I is exported to the cell surface with only a small fraction of the molecules retained inside the cell and accumulating at the cis-Golgi.

I could see the move of the D\textsuperscript{b}-GFP class I molecules to the cell surface only upon the electroporation of the FAPGNYPAL peptide. Is this effect specific for this peptide? I have introduced DNA encoding for class I and the peptide at the same time, and peptide was present inside the cell before the D\textsuperscript{b}-GFP was being expressed. Previous studies have shown that peptides after their generation by the proteasome are subjected to rapid degradation by cytoplasmic aminopeptidases, and that less than two percent of peptides are transported by the TAP transporter to the endoplasmic reticulum. In the ER, peptides then undergo further degradation by ER aminopeptidases to acquire the appropriate length for binding to the class I molecules. The half-life of the peptides has been determined as just a few seconds (for example, seven seconds for the FAPGNYPAL peptide: Reits et al., 2003). Peptides are transported by the diffusion and in the same publication, they needed six seconds to travel across the cell (Reits et al., 2003). But since their movement seems to be random, it is likely that they are degraded...
before they encounter TAP and are translocated into the ER. These findings apparently contradict my results, which show an effect of the FAPGNYPAL peptide on the D\textsuperscript{b}-GFP surface stabilization at 14-16 hours after the electroporation of the peptide. What happens with these peptides after they enter the cell and before are bound to H-2D\textsuperscript{b}-GFP molecules? I assume that a small pool of peptides is protected against degradation, probably by binding to some other molecules and then gradually released, since I can see the increase in the cell surface expression of D\textsuperscript{b}-GFP molecules over time. What are the molecules to which peptides can bind and be protected against the action of peptidases? It was reported that peptides bind to the heat shock proteins, hsp70 and hsp90, and thus become stabilized (Binder et al., 2001). Bound to the chaperones, they may be channeled from the proteasome to the TAP and then rapidly translocated into the ER. This would prevent their degradation by the cytosolic peptidases. Peptides could be also directly delivered from the proteasome to the TAP if the proteasome and the TAP transporter are connected or in the close proximity. In addition, Jacques Neefjes and collaborators have shown that peptides can bind to chromatin, namely histones H2B and H4. These peptides diffuse freely through the cytoplasm and between the cytoplasm and the nucleus. To demonstrate that the effect I can see is specific to the FAPGNYPAL peptide electroporation, I propose to use FAPGNYPAL peptide modified in the way that is either fluorescent or radiolabelled, and also posses a glucosylation consensus sequence. To make the peptide fluorescent, I will exchange the C-terminal leucine for a lysine, and attach the Cy3 or TAMRA fluorophore to it (FAPGNYPAK-Cy3 or –TAMRA). I can also use SIINFEKL-Cy3 or –TAMRA. This peptide will be
electroporated into the cell, and its localization will be observed over the time period of the above experiment (12-24 hours) by fluorescence microscopy. At different time points, I will also determine how much of the peptide is translocated to the ER. For this purpose I will change the second proline to tyrosine and attach iodine at this position (FAPGNY(125-Iodine)TAL). At different time points after electroporation, I will precipitate the total cell lysate with concanavalin A (which binds sugar moieties) and measure the radioactivity. This experiment will demonstrate the peptide survival inside the cell.

4.2. The peptide effect on class I export from the ER, and the class I ER export signal

Class I molecules need to bind high-affinity peptide in order to be stable at the cell surface, but not to reach it in the first place (Machold et al., 1995). As I and others have found, class I molecules do not need optimal peptides to leave the endoplasmic reticulum (see above). Class I molecules that bind no, or perhaps low-affinity, peptides can still be packaged into COPII vesicles and leave the ER. This makes it clear that the binding of peptide does not serve as an export signal for leaving the ER.

What then is the ER export signal for class I molecules? Do they contain one at all, or do they move to the ERGIC by bulk flow (Schekman and Orci, 1996)? It has been proposed that there are export signals in the class I amino acid sequence (Williams et al., 1985, Spiliotis et al., 2000). If they do exist, then they are probably located in the transmembrane or lumenal domain, since the attachment of GFP to the cytosolic tail of MHC class I molecules does not block
their export from the ER, and has no effect on the rate of EndoH acquisition (Tarentino and Maley, 1974; Tarentino et al., 1974). This means that such intramembrane or lumenal export signals would have to interact with the COPII coat through an intermediary protein. They also have to be unaffected by the binding of peptide since the export of both peptide-bound and peptide-receptive class I molecules from the ER is possible with approximately equal efficiency (Garstka et al., 2007).

One ER export process that may be peptide-dependent is the transport of free heavy chain with electroporated peptide that I have shown (Figure 10). It is possible that free heavy chain is retained in the ER, and that binding of peptide liberates it from stringent ER retention, allowing it to enter COPII vesicles; however, in the literature, there are several pieces of evidence that support cycling between ER and Golgi of free heavy chain (Raposo et al., 1995), which would suggest that even for free heavy chain, peptide binding is not a determinant for ER exit.

4.3. The features of peptides that determine the exit of the peptide-class I complex to the cell surface

I next wished to identify the features of the peptide that are recognized by the quality control machinery, directly or indirectly via the conformational change of class I, and that allow class I to proceed to the cell surface. First, I determined the rate of the export of H-2D\(^b\)-GFP molecules complexed with FAPGNYPAL peptide to the cell surface, and I found that after 24 hours, all class I were at the cell surface. Using this peptide as positive control and an interval of 24 hours after electroporation as sufficient for cell surface export of the class I, I investigated
a number of peptides derived from FAPGNYPAL. It is known that both the
termini and the anchor residues of the peptide are important for its binding to
class I. For H-2D\textsuperscript{b}-GFP, asparagine (N) at position five and a large hydrophobic
amino acid, for example leucine (L), at position nine, are anchor residues. I have
investigated peptides which have one anchor residue changed to the other amino acid (FAPGNYPAA), which are missing one anchor residue (FAPGNYPA),
which are missing two or more C-terminal residues (FAPGNYP, FAPGNY,
FAPGN), and which are missing four N-terminal residues (NYPAL). All these
variant peptides were shown to bind to H-2D\textsuperscript{b} \textit{in vitro}, albeit with reduced affinity
when compared to FAPGNYPAL (Tigan and Springer, not shown). These data
show that the C-terminus of the peptide is required for class I export to the cell
surface, since NYPAL can move H-2D\textsuperscript{b}-GFP molecules to the cell surface, while
FAPGNYP cannot, as compared to the sample without peptide. In addition, the
C-terminus is more important for class I export to the cell surface than the
N-terminus, since the NYPAL peptide, that lacks four amino terminus residues,
can move class I to the cell surface, while the FAPGNYP peptide, which is
missing only two C-terminus residues, cannot. At this time, other interpretations
(for example that NYPAL has a catalytic effect on the binding of endogenous
high-affinity peptide, and that it therefore supports class I surface transport) are
also valid but have not been investigated experimentally.

This inefficiency of FAPGNYP is surprising, since measurements of the
heat stability of class I-peptide complexes by differential scanning calorimetry
and tryptophan fluorescence have shown that the class I-FAPGNYP complex is
actually more stable ($T_M \approx 37.5 \, ^\circ C$) than that of NYPAL ($T_M \approx 30.5 \, ^\circ C$) (data from
tryptophan fluorescence melting curves of refolded bacterial H-2D\textsuperscript{b}, Tigan and Springer, not shown).

![Figure 19](image1.png)

Figure 19. The structure of the peptide binding groove of H-2D\textsuperscript{b} with the FAPGNYPAL peptide (A, sequence taken from Protein Data Bank, number 1CE6) B, predicted structure of the binding groove without the peptide, C, predicted structure of the binding groove with the NL5 peptide, D, predicted structure of the peptide binding groove with FP7 peptide. The pictures were done by Florian Sieker.

One possible hint comes from the published observation that the class I complex with NYPAL can be crystallized, and that the structure of the peptide binding groove of the D\textsuperscript{b}-NYPAL complex is identical to that with FAPGNYPAL peptide (Glithero et al., 2006). In contrast, it was not possible to obtain a crystal structure for the complex with FAPGNYP (Glithero et al., 2006), suggesting that its structure or flexibility may be significantly different from the FAPGNYPAL complex. In molecular dynamics simulations, the part of the peptide binding
groove where the C-terminus of the peptide is bound appeared more flexible when the peptide is missing (Petrone and Garcia 2004, Sieker et al., 2007; Zacharias and Springer 2004). On the basis of these observations, I propose that the structure of the C-terminal end of the binding groove of the H-2D\textsuperscript{b} molecule bound with FAPGNYP peptide is mostly identical to the situation when the peptide is missing (Figure 19), and that it is mostly flexible in nature as observed in the simulations.

4.4. The effect of tapasin on class I export to the cell surface

The above experiments with different peptides have shown that the carboxy terminus of the peptide is required for class I export to the cell surface. Since it binds to that part of the binding groove that is close to the proposed tapasin binding site, it is conceivable that tapasin is the molecule that detects the presence of the peptide and the strength of its binding to class I molecules. But tapasin cannot be the only factor that senses peptide loading and retains class I since in tapasin-deleted cells, class I molecules with low-affinity peptides are retained inside the cell (Sadasivan et al. 1996). If it can indeed sense the bound peptide, tapasin may help to exchange a low-affinity peptide for a higher-affinity one.

In addition, tapasin may have an additional role in the localization of peptide-receptive class I molecules inside the cell. Since peptide-receptive class I molecules cycle between ER and the \textit{cis}-Golgi, tapasin might accompany class I in COPII vesicles and itself cycle through the \textit{cis}-Golgi. Indeed, tapasin has been demonstrated to interact with the coat of COPI vesicles via its KKXX signal, and
it can be found, to some extent, in cis-Golgi membranes (Ortmann et al., 1997, Paulsson et al., 2002).

To determine whether tapasin plays a role in the trafficking of class I molecules, I have investigated the distribution of H-2D\(^{b}\)-GFP molecules in mutant cell lines that are deficient in tapasin function. I have confirmed the previous finding that H-2D\(^{b}\)-GFP is strongly dependent on tapasin for cell surface expression at steady state (Grandea et al., 2000; Suh et al., 1999; Zarling et al., 2003), since in TPN\(^{-}\) mouse ear fibroblasts (MEF), H-2D\(^{b}\)-GFP is not present at the cell surface to the same degree as in wild type MEF. As discussed previously in the literature, this defect could be due either to the lack of an essential function that tapasin has upon peptide optimization, or to the inefficient interaction of H-2D\(^{b}\)-GFP with the loading complex, since it was shown that in tapasin-negative cells, there is a decrease in the TAP, ERp57 and calreticulin found in the loading complex (Dick et al., 2002; Hughes and Cresswell, 1998; Solheim et al., 1997).

To check whether the absence of D\(^{b}\) at the cell surface in TPN\(^{-}\) MEF is caused by inefficient peptide optimization, I supplied an excess (300 nM) of optimal peptide to these cells by electroporation, but the results are not as for CHO and Vero cells, where electroporation with high-affinity peptide moved the majority of H-2D\(^{b}\)-GFP molecules to the cell surface. Instead, in tapasin-deficient cells, the supply of an excess of optimal peptide had only a partial effect on the cell surface expression of class I (Figure 15). Not all H-2D\(^{b}\)-GFP molecules were exported to the cell surface, as in CHO and Vero cells, and the distribution of overexpressed MHC class I molecules in TPN\(^{-}\) MEF cells upon electroporation of the peptide differs from the their distribution in wild type cells. While in wild
type cells class I is distributed uniformly at the cell surface 24 hours after transfection with H-2D<sup>b</sup>-GFP and peptide (or even without peptide), in tapasin-deficient cells class I is only partially present at the cell surface.

These results can be interpreted in the following ways:

1. Tapasin acts in peptide optimization. After electroporation, there is an excess of the optimal peptide, but still there are also low-affinity peptides present in these cells, and probably H-2D<sup>b</sup>-GFP can not efficiently exchange low-affinity peptides for the high-affinity ones. For this, the optimization function of tapasin is required. The small increase in the cell surface expression after the addition of the peptide in tapasin-deficient cells was because of the peptide saturation. There is more optimal peptide available for D<sup>b</sup>-GFP to bind to, and since class I can bind the peptides without the help of the loading complex, the deficiency in the tapasin function, and therefore the reduced association of MHC class I with the loading complex can be partially overcome.

MEF wild type and tapasin-deficient ones do not differ in the amount of the peptide available for binding to class I, therefore the probability of binding of this optimal peptide to class I is the same in both cell lines. Under these conditions there is strong cell surface expression of D<sup>b</sup>-GFP in wild type cells, but there is strongly reduced cell surface expression in tapasin-negative cells. There is the same amount of peptide, and the only difference is in the presence or absence of tapasin. When FAPGNYPAL peptide was added to 300 nM final concentration, it caused the increase in the concentration of optimal peptide (in both wild type and TPN-/− cells), and the probability of binding of optimal
peptide is now higher. Under these conditions, there is some cell surface expression in tapasin-negative cells, but not as for wild type cells. This would suggest that tapasin plays a role in peptide exchange. I propose that tapasin increases the speed with which peptides on class I molecules are exchanged.

2. Tapasin may be a transport chaperone for peptide-receptive class I (Paulsson et al., 2006), and therefore in tapasin-deficient cells, \(D^b\)-GFP molecules cannot proceed to the cell surface (Figure 15). However, there is evidence in the literature that in TPN\(^{-/-}\) cells, H-2D\(^b\) is still exported to the cell surface. Therefore one could propose that class I in tapasin-deficient cells is still exported to the cell surface, but at a lower rate than in wild type cells, and that the 24 hour time interval (which I used for my experiments) is not long enough. However, I have found that even after 48 hours, \(D^b\)-GFP molecules are not present at the cell surface to the same degree as in wild type cells (not shown), and it was also shown by Van Kaer and co-workers that \(D^b\) is exported to the cell surface at the same rate in both wild type and TPN\(^{-/-}\) cells, but that the surface class I molecules are not stable in tapasin-deficient cells (Grandea et al., 2000).

3. Tapasin can retain class I molecules in the ER for peptide optimization. However, then in tapasin-deficient cells, there would be faster export of the \(D^b\)-GFP molecules to the cell surface, which is contradicted by previous findings (Grandea et al., 2000).

Tapasin was shown to act in peptide optimization (Williams et al., 2002), but in addition, it may play a role in class I trafficking. Being a transport chaperone, tapasin may enter COPII vesicles together with peptide-receptive
class I molecules and become exported from the ER. Indeed, tapasin was shown to be present in post-ER compartments (Paulsson et al., 2002). If tapasin does indeed have access to COPII vesicles, optimization of the peptide could also take place in the vesicles or even at later compartment, such as the ERGIC or the cis-Golgi. I have shown that peptide-receptive class I molecules cycle between ER and cis-Golgi, and therefore one possible function of tapasin is to release forms bound with optimal peptides to the cell surface and target peptide-receptive forms for retrieval in COPI vesicles due to KKXX sequence in tapasin cytosolic tail (see above). This would explain the large amount of peptide-receptive mouse class I molecules at the cell surface of tapasin-negative cells in comparison to wild type cells.

4.5. The effect of calreticulin on class I export to the cell surface

Similar to tapasin, calreticulin has several possible roles in MHC class I peptide binding, and it is unclear how these roles relate to each other (Gao et al., Immunity 2002). First, it has been proposed that calreticulin retains the peptide-receptive forms of class I in the ER for peptide optimization (Lewis et al., 1998). Since I have shown that peptide-receptive class I molecules can leave the ER and proceed to cis-Golgi, this question has to be worded differently, namely whether calreticulin can keep peptide-receptive class I molecules in their cycle between ER and cis-Golgi. In this case, one would expect class I molecules to exit this cycle faster in calreticulin-deficient cells. Second, since calreticulin binds to the dimer of class I heavy chain and β2m in the loading complex, it may also play a role in peptide optimization, and the absence of calreticulin may decrease the
efficiency of the peptide loading and lead to the intracellular retention of class I (mediated by other factors).

To find evidence for either mechanism, I have overexpressed H-2D\textsuperscript{b}-GFP molecules in calreticulin-deficient cells, and I have found that there is a reduction of the steady state cell surface expression of class I in the absence of calreticulin in comparison to wild type cells, which confirms previous studies (Lewis et al., 1998). In addition, the distribution of D\textsuperscript{b}-GFP molecules in calreticulin-deficient cells suggests that these molecules are not stable in the absence of calreticulin, and that they are targeted for degradation. This distribution pattern suggests that in CRT\textsuperscript{-/} cells, class I is either retained in the ER and then undergoes ER associated degradation, or that these molecules in their peptide-receptive state are exported to the cell surface, but are not stable there. They are endocytosed and targeted for degradation. To stabilize H-2D\textsuperscript{b}-GFP molecules in calreticulin-deficient cells, I used the peptide electroporation assay to deliver an excess of the optimal peptide into these cells. The resulting increase of D\textsuperscript{b} surface expression shows that the delivery of high-affinity peptide can partially rescue cell surface expression of H-2D\textsuperscript{b}-GFP in absence of calreticulin.

Comparing the pattern of class I distribution in CRT\textsuperscript{-/} cells with and without high-affinity peptide, from ER distribution and accumulation (probably in lysosomes) to the subtle cell surface stain, and class I accumulated close to the nucleus, probably in Golgi apparatus, respectively, even without checking for colocalization with different organelle markers, I assume that in the absence of calreticulin, class I is rapidly exported to the cell surface in its peptide-receptive state. When high-affinity peptide was electroporated into K42 cells, the ratio of
the high-affinity peptides to the low-affinity peptides in these cells increased and therefore there was more optimal peptide available to bind to class I. Under these conditions, only a small pool of Db-GFP molecules can be stabilized by FAPGNYPAL at the cell surface. This suggests that there was no factor that would retain the Db-GFP molecules in the ER or at later compartment allowing for the peptide optimization. Therefore the proposed role for the calreticulin is to retain class I inside the cell for the peptide editing.

4.6. The role of the components of the loading complex in peptide optimization and class I trafficking

The comparison of the trafficking of class I in the different mutant cells allows interesting comparisons between the functions of the members of the loading complex.
<table>
<thead>
<tr>
<th>Loading complex component</th>
<th>Deletion phenotype</th>
<th>Peptide effect on the deletion mutant cells</th>
<th>Possible function of the component on MHC class I</th>
</tr>
</thead>
</table>
| Tapasin                   | H-2D<sup>b</sup>-GFP is strongly reduced at the cell surface | H-2D<sup>b</sup>-GFP bound with optimal peptide is present at the cell surface, but not to the same degree as in wild type cells | Optimization of the peptide cargo  
Transport chaperone |
| TAP                       | H-2D<sup>b</sup>-GFP is retained inside the cell | No effect, since peptide, in order to be loaded onto class I, needs to be delivered to the ER in a TAP-dependent manner | Delivery of the peptides that bind to the class I |
| Calreticulin              | H-2D<sup>b</sup>-GFP is strongly reduced at the cell surface | H-2D<sup>b</sup>-GFP bound with optimal peptide is present at the cell surface, but not to the same degree as in wild type cells | Retention of peptide-receptive class I molecules inside the cell |

I propose that tapasin and calreticulin work together on both peptide optimization and the trafficking of class I molecules to the cell surface. Class I in the loading complex binds to tapasin and to calreticulin and can undergo peptide optimization, i.e. exchange of low-affinity peptides for better ones over time (Williams et al., 2002). It is not known where peptide editing takes place, but since TAP, tapasin and calreticulin were shown to localize not only to the ER, but also to post-ER compartments (Kleijmeer et al., 1992; Paulsson et al., 2002;
Sonnich et al., 1994); it is likely that also peptide exchange can occur outside ER, in COPII vesicles or in ERGIC or cis-Golgi. This would mean that all forms of class I molecules are packaged into COPII vesicles, along with TAP, tapasin, and calreticulin. Tapasin, being a transport chaperone, might allow for the interaction of class I with the COPII coat proteins. If tapasin is mostly required for the export of peptide-receptive (and other?) class I molecules from the ER (something that we have not been able to show yet), it may even dissociate from the class I-calreticulin complex in some later compartment, perhaps in the ERGIC (this would be consistent with our data that tapasin can be found in post-ER compartment, probably in ERGIC, since it does not colocalize to the cis-Golgi: Esther Ghanem, not shown). Tapasin (bound to TAP, since TAP requires tapasin for its stabilization) will then interact with COPI coat in ERGIC due to its cytosolic KKXX signal and will be retrieved back to the ER in COPI vesicles. Class I bound to the calreticulin will instead be transported to the cis-Golgi, from where those forms that are bound to high-affinity peptides will proceed to the cell surface, while the peptide-receptive forms will be retrieved by calreticulin to the ER in COPI vesicles due to its KDEL signal. Calreticulin binds to the region of glycosylation in class I heavy chain that is located in the proximity of the peptide binding groove where C-terminal part of the peptide is bound, and therefore it could be able to detect, and bind to, the 'peptide-receptive state' of a class I molecule. If this detection depended on the flexibility of the amino-terminal end of the \( \alpha_1 \) helix of the peptide binding site, which is abolished by the network of hydrogen bonds formed by the last two amino acids of the peptide (F.Sieker and M. Zacharias, not shown), then this would also explain why D\( ^b \) complexes with
FAPGNYP were unable to leave the ER-Golgi cycle and proceed to the cell surface in my experiments. This model has to be tested experimentally.
5. Further research

I have confirmed that tapasin and calreticulin have important functions in the peptide optimization, but what about their role in the trafficking of class I molecules and the retention of peptide-receptive forms inside the cell?

To separate the role of tapasin in the trafficking from its function in the peptide editing, I will use conditions under which the effect of tapasin cannot be due to peptide binding, i.e. in TAP-deficient cells (T2 and TAP2d CHO cells). In these cells, I will determine the steady-state distribution and the access to the ERGIC and cis-Golgi of HLA-B*4402, which requires tapasin for the export to the cell surface, and B*4405, which does not. The difference between the distributions of B*4402 and B*4405 will be due to the direct effect of tapasin on the localization of B*4402. This will allow me to determine whether tapasin is involved in the retention or retrieval of peptide-receptive forms of class I molecules.

To determine the role of calreticulin in the trafficking of class I, I will use the human allele HLA-B27 and its S132K mutated form (which does not bind to tapasin, but still binds to calreticulin) and express them in TAP-deficient cells. Since the S132K mutation prevents the interaction with tapasin, the localization of the B27 mutant in peptide-deficient cells will be due to the specific function of calreticulin. To look for the function of calreticulin in the recycling of class I molecules, I can also use 721.220 cells that stably express ICP47. These cells do not express tapasin, and there is a reduction in the peptide supply to the ER for the binding to the class I due to TAP inhibition by ICP47.
To investigate the role of other components of the loading complex I will investigate the expression of class I in ERp57-deficient cells, and I will also try to identify the residues that are important for ERp57 binding to the class I, tapasin, and calreticulin.

The proposed experiments will allow me to elucidate the role of chaperone molecules in the trafficking of the class I molecules, and to follow up on the hypothesis presented in the discussion.
6. References


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10/1998 - 09/2003 Technical University of Lodz, Lodz, Poland
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02/2001 - 06/2001 University of the Basque Country, Bilbao, Spain
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04/2004 to date  Laboratory of Prof. Sebastian Springer, Jacobs University Bremen, Bremen, Germany
PhD Thesis "Peptide-dependent trafficking of MHC class I molecules"
Investigation of MHC class I trafficking and the retention of peptide-receptive forms inside the cell.
- Mammalian cell culture
- Immunolabeling and confocal laser scanning microscopy, image processing
- Cloning and generation of fluorescent constructs
- Subcellular fractionation by centrifugation
- Cell culture, protein production and purification (bacterial and yeast)
- Western and immunoblotting
- FRET measurements
- Flow cytometry

09/2006 - 12/2006  Collaboration with Prof. Rainer Duden, Royal Holloway, University of London, United Kingdom
Project "Molecular and cellular mechanisms of tapasin-dependent MHC class I localization"
- Mammalian cell culture
- Expression of fluorescent constructs in mammalian cells, immunolabeling
- Confocal laser scanning microscopy
- Live cell imaging

05/2005 to date  Collaboration with Prof. Mathias Winterhalter, Jacobs University Bremen, Bremen, Germany
Participation in Volkswagen Foundation project "Nanoengineered polymer capsules: Tools for detection, controlled delivery and site specific manipulation"
- Mammalian cell culture
- Confocal laser scanning microscopy
10/2003 - 02/2004  Laboratorium of Prof. Janusz Blasiak, University of Lodz, Poland
Project "DNA damages and repair"
- Cell culture (primary cells)
- Light microscopy
- Comet assay

02/2003 - 09/2003  Laboratorium of Dr. Jacek Polak, Technical University of Lodz, Poland
Cloning of tyrosine-lyase gene to high copy number vector and determination the enzyme activity
- Cloning
- Functional assays

07/2002 - 02/2003  Laboratorium of Prof. Blasiak, University of Lodz, Poland
Participation in the projects:
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"The polymorphism in the uPA gene in breast cancer”
- DNA isolation from tissues, PCR
- Protein determination, ELISA

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02/2005 - 02/2006  Graduate Student Association, International University Bremen (IUB), Germany
Treasurer and Substitute of the Secretary General of the School of Engineering and Science
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- **Garstka M.**, "Ligand-dependent intracellular transport of MHC class I molecules", VolkswagenStiftung Summer School, International University Bremen, Germany, 2006
- **Garstka M.** (2004) MHC class I trafficking. Presentation at the 2nd Open Graduate Conference, International University Bremen, Germany

**Posters:**
- **Garstka M.**, and Springer S., What keeps empty class I molecules inside the cell? 58th Mosbacher Colloquium, Mosbach 2007 (DOI:10.1240/sav_gbm_2007_m_001723)
- Borchert B., **Garstka M.**, Al-Balushi M. and Springer S. "Ligand-dependent intracellular transport of MHC class I molecules", 29th Annual meeting of the German Society for Cell Biology (DGZ), Braunschweig, Germany 2006
- **Garstka M.**, Borchert B., Al-Balushi M., Majoul I., and Springer S. "Immunofluorescence microscopy of MHC class I trafficking", 7th Young Scientists Meeting of the German Society for Cell Biology (DGZ), Jena, Germany 2005
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- **Garstka M.** "Investigation of tyrosine phenol–lyase gene expression in E. coli recombinants". (VI Poster session of MSc Theses), P-137, Technical University of Lodz, Poland, 2003
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