1 INTRODUCTION

Upon successful infection, herpesviruses stay for a lifetime in their host. Hence, persistence of the virus requires a delicate balance between the host immune system and the evasion of it by the virus. Due to tight co-evolution during millions of years both the virus and the host have developed elaborate gene functions to cope with each other. Cells are equipped with a broad spectrum of defense mechanisms, of which the main goal is to arrest virus replication or, as a final resort, self-destruction, thereby limiting spread of infection. For the virus to survive, disseminate and finally establish a latent infection, it counteracts the immune control on many fronts.

The infected host fights the virus by two main branches, the adaptive immunity and the innate immune response. Viral infection of a susceptible cell initiates a cascade of cellular reactions establishing an antiviral state. This leads to induced transcription of multiple genes coding for, among others, cytokines and chemokines, which in turn activate further specialized antiviral mechanisms of the cell, one of which is the MHC class I antigen presenting pathway.

1.1 The MHC class I antigen presenting pathway

The major histocompatibility complex class I (MHC I) antigen presenting pathway samples the intracellular protein content on the cell surface. The errorless functionality of this pathway is of highest priority when fighting intracellular intruders and malignant tumors. If foreign (viral) or mutated (cancerous) proteins exist in the cell, these are, along with endogenous self proteins, presented on the extracellular side as peptide ligands attached to the MHC class I molecule. Specific receptors on circulating CD8+ cytotoxic T lymphocytes (CTL) recognize the antigenic ligands, resulting in destruction of the cell and activation of the immune system by release of cytokines and chemokines. Cytosolic degradation of proteins into adequate peptides is the starting point for the MHC I antigen presenting pathway. Representing a bottleneck, the dedicated transporter associated with antigen processing (TAP) delivers the cytosolic peptides into the ER (endoplasmic reticulum) lumen to reach their carrier, the MHC I molecule.

1.1.1 Protein degradation – antigen generation

The proteasome is responsible for turnover of proteins by degradation in the cytosol. The antigen presentation pathway takes advantage of the proteasomal cleavage products and presents them to CTLs. Mainly ubiquitylated proteins are recognized as substrates for the proteasomal multisubunit complex, but there are also other mechanisms known for targeting proteins for degradation. Ubiquitylation requires the interaction of three enzymes, E1, E2 and E3, that in a sequential order transfer the ubiquitin to the target protein (Schwartz and Hochstrasser, 2003). Recently it has become evident that not only "old" proteins are marked by ubiquitin, but that a possibly large fraction of proteasomal substrates originate from defective translation products and non-correctly folded proteins, so called DRiPs (defective ribosomal products) (Yewdell et al., 1996). The advantage of DRiPs for the cell becomes evident when picturing a viral infection: During acute infection, the viral replication is fast and the time it would take for the cell to present antigens from functional proteins (i.e. properly translated, folded, transported and finally degraded) would be relatively long. However, presenting antigens originating from DRiPs, the time between translation of the protein and presentation by MHC I complexes on the cell surface is substantially shorter and the adoptive immune system is alarmed much faster. Thus, the cell benefits from imprecise translation and folding of proteins.

The proteolytically active 20S core of the proteasome is a huge barrel-shaped complex built up by four rings, each consisting of either seven α or seven β subunits. The rings are arranged in the order αββα. In addition, two 19S regulators attach to the 20S complex ends and form the 26S proteasome responsible for ATP dependent degradation of ubiquitylated proteins. The expression of three additional β subunits, LMP2, LMP7 and MECL1, is induced by IFN-γ. These subunits can replace β1, β2 and β5 in the 20S core, forming the immunoproteasome (Cerundolo et al., 1995). The specificity of the immunoproteasome differs from the constitutively expressed 26S complex. The INF-γ induced subunits enhance the cutting of proteins after hydrophobic and basic residues, whereas cutting after acidic residues is reduced (Groettrup et al., 2001). This fits well with the finding that both TAP and MHC I molecules preferably bind peptides with hydrophobic residues at the C-terminal end (Driscoll et al., 1993; Uebel et al., 1997; Uebel et al., 1995). Also expression of the proteasome activator complex PA28 is increased by IFN-γ. PA28 binds to the α rings of the 20S core and induces the proteolytic activity. However, the impact of PA28 on antigenic specificity is not fully understood. Overall, this exemplifies how the cell takes advantage of normal protein

housekeeping, by using the proteasomal machinery for purposes of antiviral combat and by changing some components by IFN-γ induction, turning it into an even more specialized link in the antigen presenting pathway.

1.1.2 Trimming peptidases

The peptide products of the proteasome range from 3 to 22 amino acids (Emmerich et al., 2000; Kisselev et al., 1999). Taking into account that TAP preferentially transports 8-16mer peptides, whereas the peptides that are loaded onto MHC I molecules are optimally 8-11 residues long, it is clear that only a fraction of the generated and transported peptide pool is applicable as MHC I ligands. This raised the idea of additional trimming peptidases in the cytosol and in the ER lumen. It was shown that a large amount of peptides derived from proteasomal degradation is further degraded by peptidases in the cytosol. Antigens with N-terminal extensions were observed to be trimmed and loaded onto MHC I molecules effectively, whereas antigens with C-terminal extensions were not (Craiu et al., 1997; Stoltze et al., 1998). From this followed the characterization of aminopeptidases in the cystosol as well as in the ER lumen, e.g. the cytosolic leucine aminopeptidase (LAP), expression of which is upregulated by INF-γ (Beninga et al., 1998) and the recently described ER aminopeptidase (ERAP1) that recognizes peptides as substrates only if they are longer than 7 amino acids (Saric et al., 2002; Serwold et al., 2002). If translocation to ER is prolonged the peptides are destroyed. The endopeptidase thimet oligopeptidase (TOP) assumably plays a significant role in complete degradation of peptides (Saric et al., 2001), products of which are subsequently recycled as single amino acids for protein synthesis.

1.1.3 Peptide translocation and loading of peptides onto MHC I molecules

To avoid being fully degraded before reaching the lumen of ER the peptide must within seconds bind to TAP. It has been suggested that some chaperones can interact with cytosolic peptides and rescue them from aggressive peptidases (Kunisawa and Shastri, 2003; Srivastava, 2002). Recently it was reported that the proteasome interacts with TAP, shortening the distance and time for the peptides in the cytosol (Begley et al., 2005). The evidence is preliminary though, and further examination is necessary for confirmation of the finding. The peptide binds to TAP domains close to the ER membrane (Nijenhuis and Hammerling, 1996; Nijenhuis et al., 1996) and induces conformational changes resulting in

ATP hydrolysis (Lacaille and Androlewicz, 1998), opening of the TAP pore and translocation of the peptide into the ER lumen (van Endert et al., 2002) (see following text, 1.3).

On the lumenal side TAP is part of the MHC class I peptide loading complex (PLC) consisting of the chaperones calreticulin, tapasin, and the thioreductase ERp57 and the heterodimeric MHC I molecule built by a transmembrane heavy chain (HC) and the soluble β_2-microglobulin (β_2m) (Fig. 1.1). Upon co-translational insertion in the ER membrane folding of the HC is assisted by the lectin chaperone calnexin (Degen et al., 1992). For the formation of two intrachain disulfide bonds ERp57 is recruited by calnexin and assembly with β_2m takes place (Nossner and Parham, 1995). Upon formation of the heterodimeric complex between the HC and β_2m calreticulin is released, likely as a consequence of conformational changes (Degen et al., 1992; Nossner and Parham, 1995). The highly unstable peptide receptive complex is dependent on association with further chaperons while awaiting loading of peptide. Calreticulin, a second lectin chaperone, interacts with the open MHC I molecule forming a trimeric complex found also independently in the ER lumen but mostly in association with the peptide loading complex (Sadasivan et al., 1996; Solheim et al., 1997). In order to bring the peptide supplier TAP and the MHC I molecule in close vicinity, tapasin is absolutely required (Lehner et al., 1998; Ortmann et al., 1997; Sadasivan et al., 1996). The role of tapasin though, is not simply facilitating the physical interaction of the PLC components. This was observed with a soluble tapasin, lacking its cytosolic tail and transmembrane domain needed for TAP interaction (Lehner et al., 1998; Tan et al., 2002), which still was capable of restoring MHC I stability by peptide loading (Lehner et al., 1998). Recent observations have shown that tapasin indeed exhibits a “loadase” activity, by preferentially selecting high-affinity peptides for loading onto MHC I molecules (Howarth et al., 2004). The consequence is a more stable cell surface expression of MHC I ternary complexes, thereby promoting an effective antigen presentation to CTLs and an efficient use of accessible MHC I molecules. Stably loaded MHC I molecules are released from the peptide loading complex for transport out of the ER lumen through the classical secretory pathway. Finally exposed on the cell surface, the antigen can be recognized by the T-cell receptor (TCR), which leads to activation of the CTL and the target cell is destroyed by either apoptotic or necrotic death (Barry and Bleackley, 2002). Suboptimally loaded MHC I complexes are, if at all, presented only for a short time on the cell surface and are then endocytosed for recycling.

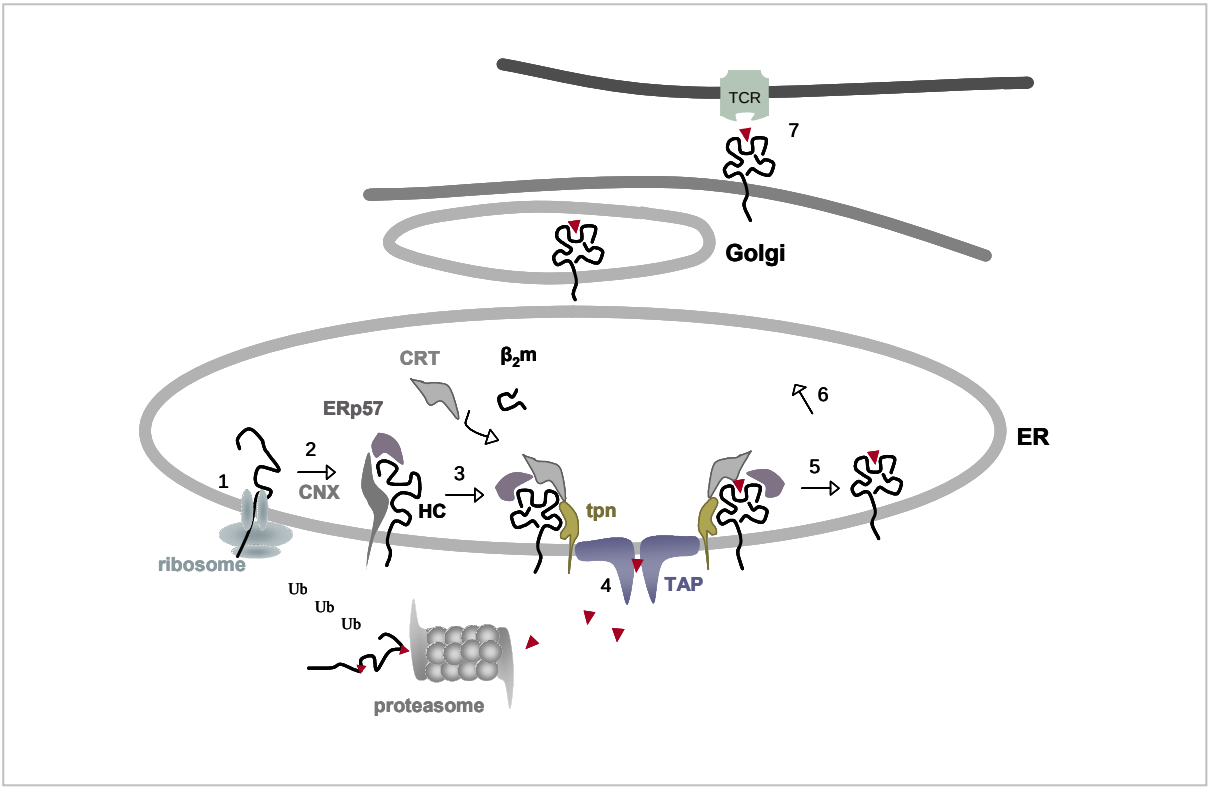

Fig. 1.1 MHC class I antigen presentation pathway.

Components of the MHC class I antigen presentation pathway are indicated in the picture, starting with insertion of the nascent HC polypeptide in the ER lumen (1). Association with calnexin (CNX) takes place and ERp57 forms disulfide bonds of the HC (2). β_2m associates with the HC with the help of calreticulin (CRT) and the the PLC forms through connection via tapasin (tpn) to TAP (3). The cytosolic proteasome degrades ubiquitinated proteins, resulting in peptides transported by TAP into the lumen of ER (4). The peptides are anchored to the peptide binding groove on the HC, the ternary MHC I molecule becomes stable (5) and leaves the ER through Golgi for cell surface expression (6). The antigen is recognized by the T-cell receptor (TCR) of the CTL (7).

The MHC class I antigen presenting pathway has developed in close contact to microbial invaders. Cytomegaloviruses possess several genes interfering with the MHC class I antigen presenting pathway. By characterizing the viral inhibitors of different components in this pathway we can learn not only how the virus protects itself from the host immune system, but by tracking the functions of the viral genes it is possible to use them as molecular tools for an expanded understanding of the cell biology of antigen presentation.

1.2 The human cytomegalovirus, HCMV

Herpesviruses have high a prevalence; from most analyzed mammalian species at least one member of the herpesvirus family has been isolated. The number of human herpesviruses is eight: herpes simplex virus type 1 and 2 (HSV-1, -2) and varizella zoster virus (VZV) belong to the α-subfamily, human cytomegalovirus (HCMV) and human herpesvirus 6 and 7 (HHV-6, -7) belong to the β-subfamily and Epstein Barr-virus (EBV) and human herpesvirus 8 (HHV-8) belong to the γ-subfamily. All herpesviruses have a similar virion structure, consisting of an icosahedral capsid surrounding the large doublestranded DNA, the tegument, a protein-filled space between the capsid and the envelope, which encloses the virus particle.

HCMV (formal designated human herpesvirus 5) is the prototype virus of the β-subgroup. The unique long (UL) and unique short (US) sequences, comprising the majority of the HCMV genes, are flanked by terminal or internal repeats called TRL, IRL, TRS or IRS, depending on their position in relation to the UL and US sequences. The repeats mediate the inversion of the unique sequences, which results in four genomic isomers. The AD169 lab strain of HCMV has been completely sequenced. It is 230 kbp long (HCMV has the largest genome of the herpesviruses) and codes for around 200 predicted ORFs (Chee et al., 1990; Murphy et al., 2003). The ORFs are given names according to their location (e.g. ORF 23 in the UL part is called UL23). The expression of the HCMV genes is tightly coordinated and can be divided into immediate early (IE), early (E) and late (L) expression kinetic.

In most cases HCMV infection leads to clinical complications only in immunocompromised individuals, like AIDS and allograft transplantation patients. The primary infection is effectively controlled by a competent immune system, but HCMV persist in the host in a latent phase. Factors causing a recurrent infection are not well understood. Due to excretion of the HCMV particle into saliva, genital fluids and breast milk, intimate contact is necessary for HCMV transmission. Vertical infection from mother to child by breastfeeding is a common mean of infection (Hamprecht et al., 2001), but also intrauterine transmission during pregnancy is possible (Fowler et al., 1992). The reported incidence of HCMV congenital infection is 0,5-2,0 % (Peckham, 1991) and is a leading cause of mental retardation and deafness of live-born infants.

HCMV is a slow replicating (one replication cycle in 72 hours) strictly species-specific virus. A wide range of animal species has also been found to harbor cytomegaloviruses, of which the mouse CMV (MCMV) is the most frequently used animal model. An animal model is essential for studying CMV biology *in vivo*, especially for understanding of immune responses and virus spread. Many gene functions are dispensable for virus replication in cell culture. Analysis of viral mutants in animal models provides understanding of the context of CMV immune and cell biology. Because of a broad cell tropism and replication in the presence of CMV-specific immune responses the virus is constantly subjected to immune pressure. In defense, a large fraction of the HCMV genome encodes proteins involved in escape of immune control.

1.2.1 HCMV inhibitors of the MHC class I antigen presenting pathway

Up to the present, there are four well characterized HCMV encoded proteins with MHC class I pathway evasive functions, shortly called immunoevasins (Reddehase, 2002). The inhibitors *US2*, *US3*, *US6* and *US11* are, as their names indicate, encoded in the unique short (US) region of the HCMV genome. The genes were divided into two closely related gene families: *US2* and *US3* belong to the *US2*-gene family, whereas *US6* and *US11* belong to the *US6*-gene family (Chee et al., 1990). By exhibiting differing expression kinetics during the HCMV replication cycle reaching from IE to L, the immunoevasins ensure a durable downregulation of the antigen presenting pathway. They are all type I transmembrane glycoproteins consisting of a short cytosolic tail, one transmembrane domain and a bulky lumenal part (Gewurz et al., 2001; Kyritsis et al., 2001; Misaghi et al., 2004).

The glycoproteins US2 and US11 (gpUS2 and gpUS11) bind to MHC I HC in the ER lumen and dislocate it for proteasomal degradation in the cytosol (Wiertz et al., 1996a; Wiertz et al., 1996b) (Fig.1.2). There is strong evidence for gpUS11 facilitated ubiquitylation of the HC (Kikkert et al., 2001; Shamu et al., 2001; Shamu et al., 1999). Early investigations suggested Sec61 as a possible channel for gpUS2 and gpUS11 dependent HC dislocation (Tirosh et al., 2003; Wiertz et al., 1996b). However, the crystal structure of Sec61 shows an open pore obviously too small for high-mannose bearing HC (Blom et al., 2004; Van den Berg et al., 2004), calling for other possible channels in the ER membrane. The immunoevasins have proven to be helpful tools in investigation of mechanisms of protein dislocation out of ER. Using gpUS11, a second ER resident membrane channel was identified. Derlin-1 conducts the translocation of MHC I HC by wild type gpUS11, whereas gpUS11$_{Q192L}$ with a single amino

acid mutation in the transmembrane domain does not. gpUS2 is not capable of mediating HC translocation by Derlin-1, pointing at a different mechanisms of HC removal from the ER than applied by gpUS11 (Lilley and Ploegh, 2004; Ye et al., 2004).

The glycoprotein US3 (gpUS3), even if possessing a similar structure and an Ig-like domain as gpUS2 (Gewurz et al., 2001; Misaghi et al., 2004), utilizes a different mode of action in order to prevent MHC I molecules from leaving the ER. gpUS3 interacts with MHC I molecules transiently, thereby retaining them in the ER without disturbing peptide loading (Ahn et al., 1996a; Jones et al., 1996) (Fig. 1.2). Mutations leading to loss of retention of gpUS3 still do not disrupt interaction to MHC I molecules (Lee et al., 2003). Furthermore, the half-life of gpUS3 is shorter than retained MHC I molecules (Gruhler et al., 2000). Recently, it was published that gpUS3 forms oligomers within the MHC I complex (Misaghi et al., 2004), offering a more specific model of MHC I retention. gpUS3 molecules can be switched out of the MHC I–multi-gpUS3 complex with a high frequency without letting the MHC I molecule escape retention. The oligomeric form of gpUS3 offers more possibilities to interact with chaperons containing a retention signal, which is not found in the gpUS3 sequence itself. Also a second target in the MHC class I pathway has been accredited to gpUS3. Following the observation that only tapasin-dependent class I alleles were affected by gpUS3, whereas tapasin-independent MHC class I escaped ER retention, direct gpUS3 binding to tapasin and inhibition of optimal peptide loading was verified (Park et al., 2004).

Furthermore, both gpUS2 and gpUS3 have been described as inhibitors of the MHC class II pathway. It was published that gpUS2 binding to HLA-DR-alpha and HLA-DM-alpha MHC II chains leads to degradation of the molecules, whereas gpUS3 interaction to class II alpha/beta complexes inhibits the interaction with the invariant chain (Hegde et al., 2002; Johnson and Hegde, 2002).

In addition, a sequence homologue to the inhibitors portrayed above, gpUS6, targets a different component of the MHC I antigen presenting pathway. gpUS6 binds to the peptide transporter TAP and turns off the translocation cycle (Fig. 1.2).

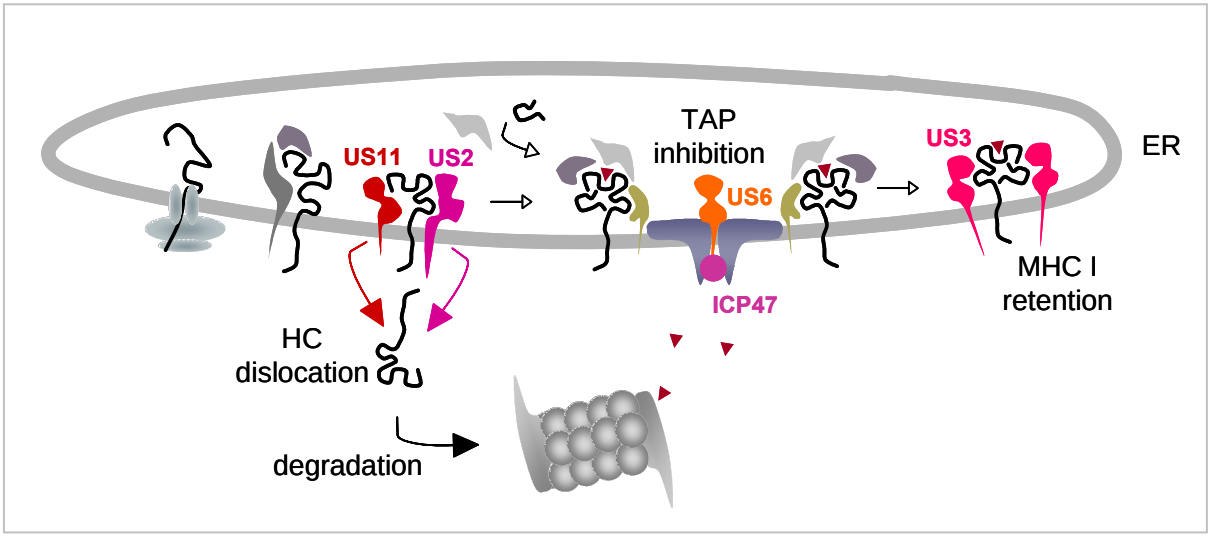

Fig. 1.2 Herpesviral inhibitors of the MHC class I antigen presenting pathway.

HCMV encoded glycoproteins US2 and US11 bind to the HC and dislocate it from the lumen of ER to the cytosol for proteasomal degradation. HCMV glycoprotein US3 interacts with the MHC I molecule and prevents it from leaving ER for cell surface expression. TAP inhibitors gpUS6 and ICP47 bind to the peptide transporter TAP and prevent peptides from being translocated into the ER. For identification of single components of the MHC class I antigen presenting pathway see Fig. 1.1.

1.3 The transporter associated with antigen processing, TAP

1.3.1 TAP is an ABC transporter

The peptide exporter TAP is a member of the ABC (ATP binding cassette) transporter family, one of the biggest paralogous protein families with representatives found in all branches of life, i.e. prokaryoa, eukaryoa and archaea. The first studied ABC transporters were of bacterial origin. As the cause of inheritable human diseases like cystic fibrosis, adrenoleukodystrophy and Stargardt's disease was pinpointed to mutations in certain ABC transporters (Allikmets et al., 1997; Azarian and Travis, 1997; Collins, 1992; Mosser et al., 1993), the interest for mechanisms and structure of this diverse class of protein family increased extensively. ABC proteins translocate a wide spectrum of substrates, such as sugars, amino acids, ions, vitamins and peptides across lipid membranes. The tranporters are built by four domains, two transmembrane domains (TMDs) usually consisting of a core of six transmembrane segments each, and two nucleotide binding domains (NBDs). The four domains can be expressed as a single polypeptide chain or as separate polypeptides fused in a number of ways in order to form the transmembrane pore with the attached NBDs (Higgins, 2001).

Not much is known about human genetic TAP defects. The human genetic disease Bare lymphocyte syndrome (BLS) is caused by a premature stop codon in the TAP2 gene, leading to severe downregulation of MHC I complexes from the cell surface (de la Salle et al., 1994; Teisserenc et al., 1997). BLS manifests with necrotizing granulomatous skin lesions and recurrent bacterial infections. In addition, deficient TAP has been observed in breast cancer (Alimonti et al., 2000) and small lung cancer (Seliger et al., 1997). Reduced levels of MHC I surface expression supports the escape from CTL recognition of the tumoral cell. In patients suffering from autoimmune diseases downregulation of TAP1 and TAP2 mRNA levels has been reported (Fu et al., 1998). Cells lacking MHC I molecules activate natural killer cells and promote the autoimmune disease.

Substrate translocation requires opening of the transmembrane pore, a rearrangement of the transmembrane helices energized by ATP hydrolysis by the NBDs. Sequence similarities of the TMDs of different ABC transporters are found only if the transporters move similar substrates in the same direction. Exporters and importers therefore utilize a common engine, the NBDs, attached to a substrate specialized translocation pore (TMDs). There are several

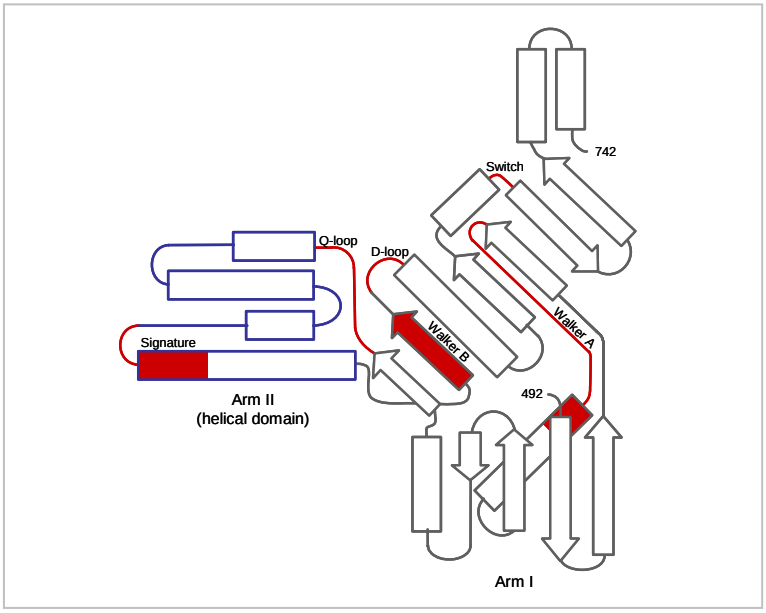

Fig. 1.3 Structure of the TAP1 NBD.
α-helices are shown as boxes, β-strands as arrows and conserved motifs among ABC transporters in red (Gaudet and Wiley, 2001).

crystal structures existing of NBDs of ABC transporters (Diederichs et al., 2000; Hung et al., 1998; Yuan et al., 2001), including that of TAP1 (Gaudet and Wiley, 2001), whereas a complete structure is available only for three bacterial ABC transporters, the *E. Coli* and *V. Cholera* lipid A exporter MsbA (Chang, 2003; Chang and Roth, 2001) and the *E. Coli* vitamin B12 importer BtuCD (Locher et al., 2002). The NBDs hold a very similar fold regardless of the transporter and the origin. The NBD folds in two arms, taking an L shape (Beismann-Driemeyer and Tampe, 2004; Schneider and Hunke, 1998). Arm I is composed of two beta-sheets and six alpha-helices and contains the Walker A (or P-loop) (GxxGxGKT/S; x can be varied) and B motifs (hhhhD; h stands for hydrophobic) in addition to the conserved sequence motifs, the D-loop and the switch region (Fig. 1.3). The smaller helical arm II, comprising the signature motif (or C-loop) and the Q-loop, is thought to function as a signaling domain. Residues of the signature motif and the Q-loop were found to be flexible and suggested to be stabilized and altered in location by the presence of a γ-phosphate from a nucleotide (Gaudet and Wiley, 2001). This mobility could confer important movements for signaling between the NBDs and the TMDs. The highly conserved Walker A motif of one NBD binds α- and β-phosphates of the nucleotide, whereas the signature sequence of the opposing NBD sensors the γ-phosphate, thus, the ATP molecule is fixed between the two subunits in the NBD dimer. Interestingly, the signature sequence (LSGGQ) is mutated in TAP2 (LAGGQ) and could be related to the asymmetric function of the TAP NBDs during the transport cycle (see following text). In a recent study mutations of the signature sequences of TAP1 and TAP2 revealed that the mutated sequence slows down the peptide transport, indicating that the signature motifs are controlling the transport (Chen et al., 2004). Still, it is not clear in what manner the ATP energy is transferred from the NBDs to the TMDs. The crystal structure of MsbA revealed a cone shaped TMD formed by 12 alpha-helical TMSs, with the opening of the pore directed

towards the NBDs (Chang, 2003). The structure suggests that upon ATP binding and hydrolysis the NBDs are in close contact, resulting in movement of the transmembrane helices that drives the substrate through the translocation chamber.

1.3.2 Structure and function of TAP

By chromosome walking TAP1 and TAP2 were identified as two mutated genes, responsible for impaired antigen presentation in several cell lines (Bahram et al., 1991; Spies et al., 1990). TAP1 and TAP2 locate to the ER membrane, they are functional only when co-expressed as heterodimers and ATP dependent (Kleijmeer et al., 1992; Meyer et al., 1994; Neefjes et al., 1993). Each subunit contains one C-terminal TMD and one N-terminal NBD. The human TAP1 is 748 amino acids in length with a calculated molecular mass of 81 kDa, whereas TAP2 is somewhat shorter with 703 amino acids and 75 kDa. The overall sequence homology of the two subunits is 33%, but the NBDs are highly homologous with around 80% identity.

The peptide binding site on TAP has not been completely mapped to date. It was shown that cytosolic domains close to the membrane, that are not parts of the NBD, are responsible for peptide binding (Fig. 1.5) (Arora et al., 2001; Nijenhuis and Hammerling, 1996; Nijenhuis et al., 1996). More effort has been put into the understanding of TAP selectivity for preferentially 8-16mer peptides (van Endert et al., 1994). Based on several studies using different peptide libraries it was possible to determine the sequence constraints of peptides suitable as TAP substrates (Meyer et al., 1994; Shepherd et al., 1993; Uebel et al., 1997; Uebel et al., 1995). Almost exclusively only the three first N-terminal and the last C-terminal positions bear specificities for TAP. Basic and hydrophobic residues are favorable at the C-terminal position. At the N-terminal second position proline is strongly disfavored. The internal amino acids of the peptide are promiscuous in sequence and length. Bulky side chains and labeling with fluorophors do not disturb the binding to TAP and translocation. Interestingly, the TCR mostly recognizes the

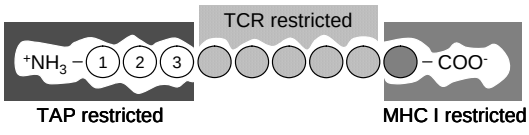

Fig. 1.4 Constraints of peptide recognition.

Peptide interaction with TAP is mainly conducted by the N-terminal amino acids in positions 1-3. Anchoring of the peptide to the MHC I molecule is restricted by the C-terminal amino acid, whereas the internal amino acid sequence is recognized by the TCR. Modified from (Beismann-Driemeyer and Tampe, 2004).

residues 5-8 of the antigen bond to the MHC molecules, i.e. TAP selectivity does not restrict the antigenic pool of peptides (Fig. 1.4).

Due to lack of crystallographic data, the number and organization of the transmembrane segments (TMSs) of TAP have not been defined. The available data based on sequence alignments, transmembrane topology predictions and one experimental approach, point at two possible models of the TAP TMDs. J. Neefjes and co-workers (Vos et al., 1999) constructed C-terminally truncated TAP mutants with inserted N-glycosylation sites. From the glycosylation pattern they proposed a topology model with 8 TMSs for TAP1 and 7 TMSs for TAP2. In contrast, R. Abele and R. Tampé (Abele and Tampe, 1999) introduced a TAP topology based on sequence alignments with the well characterized MDR (multidrug resistance) transporter P-gp. In their model two additional weakly hydrophobic α-helices are inserted in the C-terminal TMD of both subunits. Accordingly, TAP1 contains 10 TMSs and TAP2 9. In a recent study, supporting the latter model, binding domains for tapasin was specified on TAP. TAP1 and TAP2 subunits lacking four and three N-terminal TMSs, respectively, were not able to associate with tapasin, however, retained their functionality (Koch et al., 2003). As characteristic for the translocation pore of ABC transporters, the truncated TAP subunits still possessed six putative TMSs each (R.A. and R.T. topology model), thus, designed as the core TMD. Therefore, it is tempting to assume that the supplementary N-terminal TMSs of TAP1 and TAP2 evolved in order to gain a close contact to MHC I molecules (i.e. efficient peptide loading) via tapasin and are not involved in the actual peptide translocation procedure.

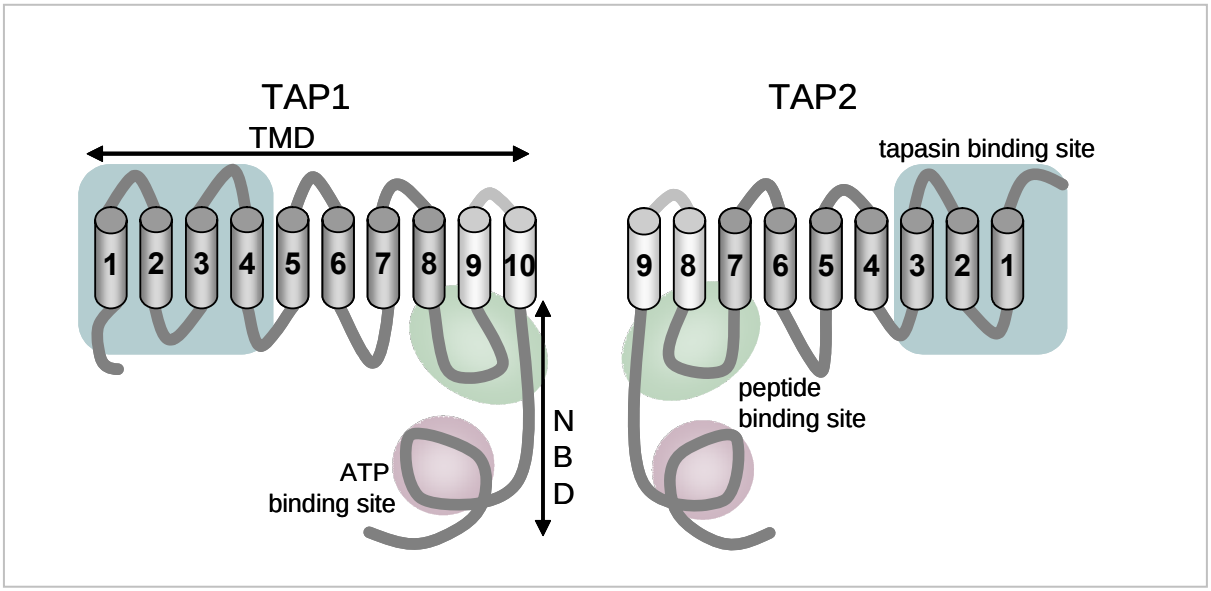

Fig. 1.5 Characteristics of the heterodimeric TAP complex.

The peptide binding and ATP binding (Walker A motif) sites are highlighted by green and pink areas, respectively. The tapasin binding sites on TAP1 and TAP2 are highlighted by blue. The controversial TMSs 9-10 of TAP1 and 8-9 of TAP2 are depicted in a light grey color.

An interesting, though complex topic of research is the order of events during one transport cycle. It was calculated that a single TAP complex hydrolyses approximately five molecules of ATP per second to transport two to three peptides (Gorbulev et al., 2001), corresponding to hydrolysis of one ATP molecule by each NBD for transport of one peptide. When incubating TAP with orthovanadate (traps the ATP hydrolysis in the transisition state ADP + V_i) subsequent ATP binding is always found at TAP1, indicating that TAP2 is trapped by vanadate (Karttunen et al., 2001). This points at an asymmetric catalytic cycle, described also for other ABC transporters (Gao et al., 2000; Senior et al., 1995). To clarify if peptide binding to TAP is ATP dependent and to understand the roles of the disparate NBDs, the key lysine of the Walker A motif was mutated. The impact of this mutation on peptide binding and transport was analyzed in several studies and different systems with partly divergent results. Mutation of the TAP1 lysine was found not to have any effect on the peptide binding in all studies and residual transport activity could still be measured (Karttunen et al., 2001; Lapinski et al., 2001; Saveanu et al., 2001). Binding of ATP to the TAP1 Walker A mutant was inconsistently described. In two studies a slow and weak ATP binding could be demonstrated (Karttunen et al., 2001; Lapinski et al., 2001), in another one no binding of ATP could be detected (Saveanu et al., 2001). Corresponding analysis of TAP2, led to equal findings concerning ATP binding to TAP1 and all studies could verify a complete loss of peptide

transport, which indicates that peptide transport is absolutely dependent on ATPase activity by TAP2 but not by TAP1. However, in contrast to TAP1, peptide binding was in two cases found to be dependent on a functional TAP2 Walker A motif (Karttunen et al., 2001; Saveanu et al., 2001). Based on these and earlier findings a model for the translocation cycle was proposed, favoring ATP-dependency at TAP2 for substrate binding (van Endert et al., 2002): The cycle starts with ATP binding to TAP2. This allows peptide binding in a high affinity state, which induces subsequent conformational changes (Lacaille and Androlewicz, 1998) and ATP hydrolysis by TAP2. This in turn, leads to ATP binding to TAP1 and further conformational changes, nucleotide release at TAP2 with subsequent reduction of peptide affinity and release of the peptide. Simultaneously ATP is hydrolyzed by TAP1, inducing rearrangements of the TMSs and displacement of the peptide on the lumenal side. Nucleotide release by TAP1 resets the cycle. Still, many of the steps in the suggested sequence of events remain to be confirmed.

1.4 Viral TAP inhibitors

Since the identification of the HSV-1 encoded TAP inhibitor ICP47 and the HCMV encoded gpUS6 additional viral functions have been found to influence TAP in a debilitating manner. In particular herpesviruses encode proteins that interfere with TAP expression or function, which might be a result of the characteristic co-existence with the host. So far, the characterized TAP inhibitors exhibit varying mechanisms of function.

1.4.1 gpUS6

Shortly after phenotypic description of arrested peptide transport in HCMV-infected cells expressing high levels of TAP (Hengel et al., 1996), three independent publications reported the inactivation of TAP by the HCMV glycoprotein US6 (Ahn et al., 1997; Hengel et al., 1997; Lehner et al., 1997). The *US6* ORF is located in the HCMV genome from basepair 195949 to 195397 (Chee et al., 1990) and is expressed with an E/L kinetic (Jones and Muzithras, 1991). gpUS6 is a 21 kDa type I transmembrane protein consisting of 183 amino acids and a single glycosylation site at the asparagine residue N52. Truncation mutants of gpUS6 have proven the lumenal part being sufficient for TAP inhibition (Ahn et al., 1997; Hewitt et al., 2001; Kyritsis et al., 2001), indicating that the TMS is dispensable for its function and is likely to serve only the attachment of gpUS6 to the ER membrane.

gpUS6 associates with the peptide loading complex and calnexin (Ahn et al., 1997; Hengel et al., 1997). Still, functional inactivation of TAP is neither dependent on tapasin nor on MHC I HC (Hengel et al., 1997), in fact, gpUS6 inhibits the peptide translocation independent of other factors (Kyritsis et al., 2001). Peptide binding to TAP is not disrupted in the presence of gpUS6 (Ahn et al., 1997; Hengel et al., 1997), rather, the inhibitory mechanism strikes TAP in a later phase of the translocation cycle. It was shown that gpUS6 interferes with the ATP binding, inhibiting binding to TAP1, whereas ATP binding to TAP2 was induced (Hewitt et al., 2001). Peptide binding induces conformational changes leading to a 210 kDa TAP complex when crosslinked (Lacaille and Androlewicz, 1998). In the presence of gpUS6 this complex does not appear, pointing at hindrance of conformational changes by gpUS6 (Hewitt et al., 2001). In a preceding study (Halenius et al., 2005) it was shown that gpUS6 is species-specific, inhibiting peptide transport by TAP only in human and monkey cells, but not in mouse and rat cells. Binding requirements on TAP was investigated and revealed that gpUS6 recognizes only preformed heterodimers of TAP1 and TAP2, i.e. gpUS6 does not interact

with single expressed subunits. Furthermore, co-expression of human and rat TAP subunits showed that gpUS6 binding sites exist on both TAP1 and TAP2.

1.4.2 ICP47

Initially identified as an HSV-1 gene product inhibiting antigen presentation to CTLs, ICP47 was the first TAP inhibitor reported (Fruh et al., 1995; Hill et al., 1995). HSV-1, in contrast to HCMV, appears to possess only this specific inhibitor of the MHC class I pathway. The primary entry of HSV leads to oral or genital infection with a fast and efficient replication and a resulting viral spread. After infection of neurons and migration to trigeminal or sacral ganglia, HSV establishes a latency state with minimal gene expression in cranial nerve ganglia or spinal ganglia, an environment with low lymphocyte circulation. For immune evasion, HSV possess several genes specialized in inhibition of IFN-responses and apoptosis (Derfuss and Meinl, 2002; Leib, 2002).

ICP47 is a small soluble protein localized to the cytosol (York et al., 1994), i.e. in contrast to gpUS6 it interacts with the cytosolic face of TAP. Accordingly, gpUS6 and ICP47 exhibit completely different molecular mechanisms of TAP inhibition. ICP47 of HSV-1 is 89 amino acids long (8,5 kDa), but the amino acids 2-35 suffice for efficient blockade of peptide translocation (Galocha et al., 1997; Neumann et al., 1997). Two studies have been published, characterizing ICP47 binding to TAP. It was found that the affinity for human TAP is about 50nM (Ahn et al., 1996b; Tomazin et al., 1996), whereas for mouse TAP it is 100-fold lower (Ahn et al., 1996b), indicating that ICP47 is species-specific. Furthermore, ICP47 was found to inhibit peptide binding to TAP (Ahn et al., 1996b; Tomazin et al., 1996). The authors suggested that ICP47 functions as a high-affinity competitor to the peptide binding site, as it competed efficiently for TAP in comparison to single high-affinity peptides or a peptide library. However, the Scatchard analysis delivered inconsistent data, which was interpreted as ICP47 interaction with additional residues of TAP (Ahn et al., 1996b). This was emphasized by the finding that ICP47 inhibits conformational changes induced by peptide binding (Lacaille and Androlewicz, 1998), suggesting that ICP47 induces a loose TAP conformation destabilizing the heterodimer. ICP47 had no effect on ATP binding to TAP1 nor to TAP2 (Ahn et al., 1996b; Tomazin et al., 1996).

1.4.3 Further viral TAP inhibitors

The Epstein-Barr virus (EBV) is a human tumor virus that belongs to the γ-herpesvirus family. The EBV protein BCRF1, a viral interleukin 10 (vIL-10) homologue, hampers the peptide transport by reducing the TAP1 expression (Zeidler et al., 1997). HCMV also encodes a vIL-10 gene (Kotenko et al., 2000), however, it has not been put into direct relevance for TAP expression. Several animal α-herpesviruses that belong to the genus varicelloviruses were found to express an early protein inhibiting TAP. Reduced levels of MHC I surface expression and impaired peptide transport by TAP was observed in cells infected by pseudorabies virus (PrV) (Ambagala et al., 2003; Ambagala et al., 2000), by Bovine herpesvirus 1 (BoHV-1) (Koppers-Lalic et al., 2003) and by Equine herpesvirus 1 (EHV-1) (Ambagala et al., 2004). The responsible gene though, has only been identified for BoHV-1 (Koppers-Lalic et al., 2005), but homologs are encoded also in PrV and EHV-1. The UL49.5 gene product of BoHV-1 binds to TAP and inhibits translocation of peptide. Furthermore, the cytoplasmic tail of UL49.5 confers proteasome dependent degradation of TAP (Koppers-Lalic et al., 2005). The murine gamma-herpesvirus-68 (MHV-68) protein MK3 was first described as an inhibitor of the MHC I molecule, by binding to it and directing it for proteasomal degradation (Boname and Stevenson, 2001). Recently MK3 was subsequently characterized as a RING finger dependent mediator of TAP and tapasin degradation (Boname et al., 2004). MK3 binds to TAP1 independently of TAP2 and tapasin. In the absence of TAP1, MK3 was unstable, reflecting that the MK3-TAP1 interaction stabilizes MK3. The only non-herpesviral TAP inhibitor that has been described is the adenoviral E19 protein. E19 binds to TAP, thereby inhibiting the significant interaction to tapasin and bridging to MHC I molecules (Bennett et al., 1999). Except for this function E19 was reported to bind MHC I molecules and inhibit their cell surface expression (Burgert and Kvist, 1987).

1.5 Aim of the study

The superfamily of ABC transporters constitutes proteins transporting a broad spectrum of substrates across biological membranes. Because of their multifunctional composition, including the pore forming transmembrane domains and energy supplying nucleotide binding domains, structure and function are difficult to study and not fully understood. To date biological inhibition of ABC transporters is limited to very few herpesviral proteins. gpUS6 of HCMV and ICP47 of HSV-1 represent specific inhibitors developed to block the TAP dependent transport of peptides at different stages (Momburg and Hengel, 2002).

The aim of this study was to elucidate physical and functional interactions of the inhibitors with TAP in order to gain insight into molecular aspects of TAP structure (e.g. membrane topology) and function (e.g. coupling of NBD and TMD). A common feature of gpUS6 and ICP47, the species restricted inhibition of TAP, was to be exploited. Using newly designed interspecies TAP hybrids, this project intended to define the minimal binding domains on TAP for interaction with gpUS6 and ICP47 and inhibition of TAP mediated peptide translocation. Furthermore, interrupting the peptide transport cycle by mutating the ATPase activity of TAP1 and TAP2, conformational constraints on TAP were to be evaluated. By broadening the understanding of TAP inhibition mechanisms and mode of action, future projects developing inhibitors for other ABC transporters, such as the MDR transporter P-gp responsible for export of anticancer drugs out of the cell, might benefit from new perceptions.