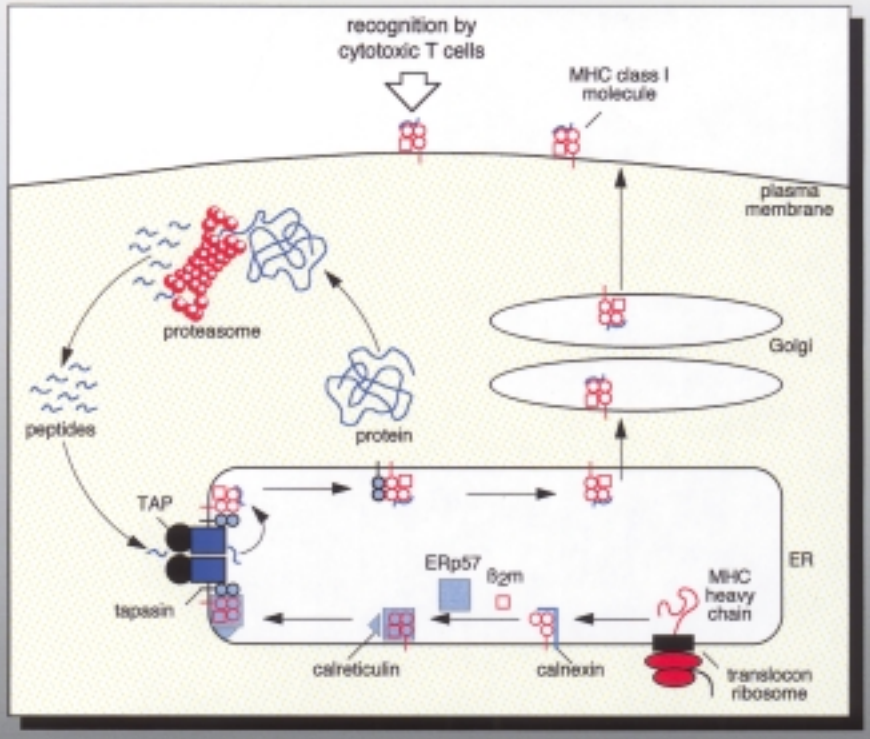
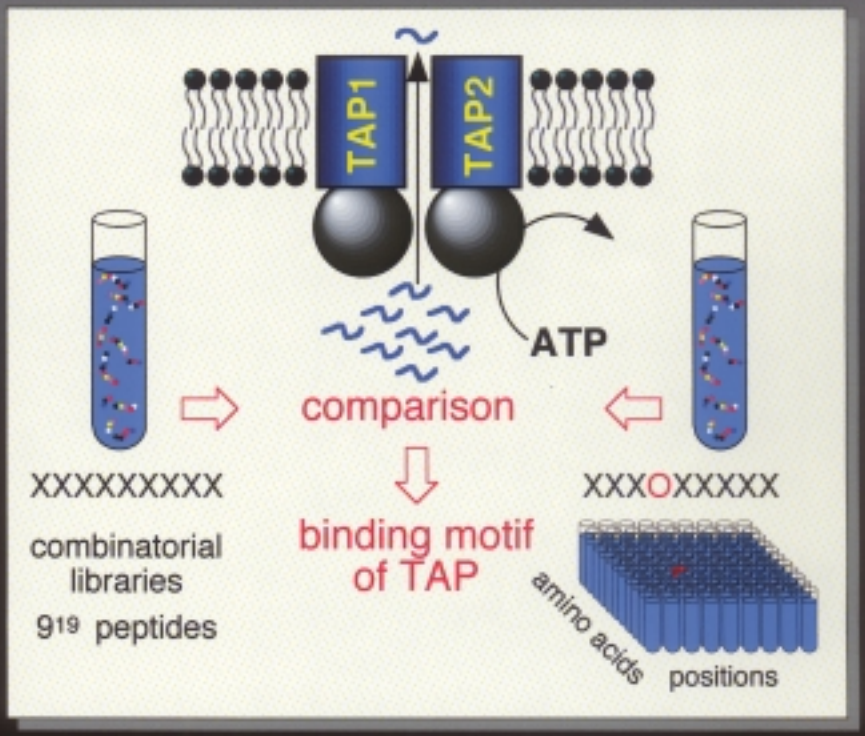
At the crossroad of immunology, cell biology, membrane and combinatorial biochemistry ...



Turning the inside out – function of the translocation machinery TAP in cellular immune recognition



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Affinity, Specificity, Diversity: A Challenge for the ABC Transporter TAP in Cellular Immunity

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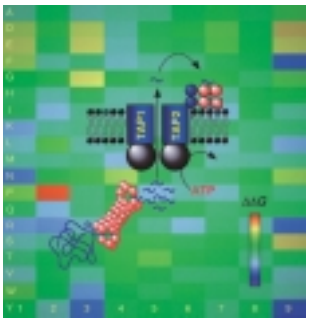
The immune system is a perfect piece of machinery that serves only one purpose: to protect and defend our organism. The fact that we are still alive, although surrounded by a hostile environment, demonstrates impressively how well the immune system performs this task. Over the past decades our knowledge about the functions and components of the immune system has increased exponentially. Especially for the humoral immune system and its key players, the antibodies, a detailed knowledge about structure and function as well as regulation and communication between the individual components exists. In case of the cellular immune system that knowledge is not as profound as for the humoral counterpart. In this article we do not intend to give a complete overview about both branches of the immune system. Rather, we want to focus on an essential protein of the cellular immune system, the transporter associated with antigen processing (TAP). This transmembrane protein complex displays unique properties with respect to recognition and translocation of a vast spectrum of antigenic peptides. Its important role within the major histocompatibility complex (MHC) class I mediated immune response has been utilized by some viruses which target TAP to hide and escape from a deadly attack by the immune system. Here we summarize the existing data and analyze structural and mechanistic aspects of recognition and transport by which this transporter performs its task.

KEYWORDS:

combinatorial chemistry · membranes · molecular recognition · peptides · proteins

1. The function of TAP in cellular immune surveillance



Our organism is confronted daily with billions of dangerous and sometimes even deadly pathogens. The spectrum of these invaders ranges from small organic and inorganic molecules to viruses and bacteria. All of these compounds are recognized and eliminated because they bear structural elements that are recognized as "nonself". Everything else, which is correspondingly called "self", has to be tolerated by the adaptive immune system.[1–4] To ensure this task, a process, which is called negative and positive selection, occurs in the thymus for T-cell selection[5] and in the bone marrow for B-cell selection,[6] respectively. In these tissues all elements of the immune system that recognize "self" elements are eliminated (negative selection), while the structural elements tolerating "self" elements migrate into the periphery (positive selection). The exact mechanisms involved in these selections as well as the molecular events resulting in the discrimination are not fully understood yet. Nevertheless, our immune system is able to recognize, at least in theory, every possible three-dimensional structural motif not present in our own organism and to consequently eliminate it.

The adaptive immune system can be divided into two lines of defense, the humoral[7] and the cellular systems.[8] The cellular immune system can be further subdivided into the class I major histocompatibility complex (MHC) and the class II MHC pathways.[9] The humoral immune system employing antibodies as search engines is able to recognize, label, and finally eliminate a wide variety of foreign particles such as proteins, carbohydrates, or fatty acids, which are located in the body fluid and the cell surface of the invader. But any invader that is able to hide within a cell can thwart this defense line. Thus, a cellular immune system has evolved to ensure that infected cells can be recognized and destroyed.

1.1. A brief introduction to the cellular immune system

Probably one of the striking examples of the failure of the humoral immune system is leprosy. This disease is caused by the pathogen *Mycobacterium leprae* infecting macrophages. As soon as the bacteria are hidden inside the macrophages, they are outside the reach of antibodies which consequently fail to prevent the disease. On the other hand, activation of the cellular branch of the immune system protects the host against the onset of the disease. Using MHC molecules that present fragments of proteins (antigenic peptides) to the T-cell receptor

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(TCR) of T lymphocytes,[10] the cellular immune system is able to monitor events inside a cell (Figure 1). Thus, in principle even a hidden, intracellular pathogen can be detected and the host cell will be recognized as infected, thus foreign ("nonself"), and finally be destroyed. Because MHC-bearing cells present antigenic peptides, they are called antigen-presenting cells (APC).[11] In general, antigen presentation refers to the fact that antigenic epitopes (peptides) are presented at the cell surface to the TCR.[1]

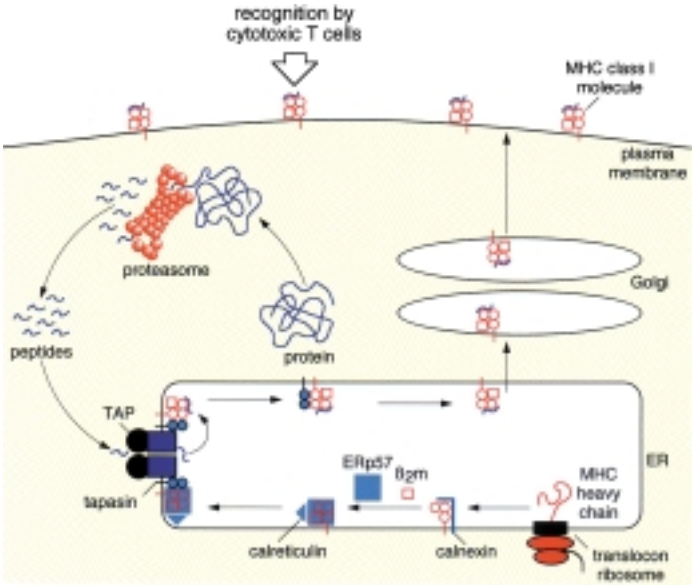

Figure 1. *The role of TAP in antigen processing and presentation. The proteasome, a multicatalytic protease complex, degrades cytosolic proteins. TAP transports these fragments into the lumen of the ER. Here peptides are loaded on nascent MHC class I molecules, assisted by a complex network of chaperones. Subsequently, matured class I MHC peptide complexes traffic to the cell surface, where their antigenic cargo is screened by $CD8^+$ T lymphocytes.*

Therefore, antigen processing refers to all processes inside the cell that finally lead to the presentation of peptides at the cell surface. Thus, degradation of proteins, intracellular transport of the peptides generated, loading of these onto MHC molecules, and the subsequent trafficking of MHC – peptide complexes to the cell surface are processes summarized under antigen processing. All events occurring at the cell surface of APC, including interaction with and recognition by TCR, therefore belong to the overall process of antigen presentation.

The binding of antigenic peptides to the MHC molecules, stable complex formation, and subsequent presentation of the MHC – peptide complex at the cell surface result in a read-out module for the TCR.[12, 13] The same principles of self vs. non-self discrimination as for antibodies apply for the TCR. Whereas MHC molecules can bind and present a very large repertoire of peptides, TCRs only recognize nonself MHC – peptide complexes outside the thymus. Thus, only in the case of a nonself peptide bound to a MHC molecule a stable ternary complex (MHC – peptide – TCR) is formed.

This ternary complex constitutes another branch point for the immune system. MHC molecules can be subdivided into MHC class I and class II molecules. Whereas class I MHC molecules

present their target to the TCR of $CD8^+$ T lymphocytes (cytotoxic T lymphocytes, CTL)[14] (Figure 1), class II MHC molecules present the bound antigenic peptide to the TCR of $CD4^+$ T lymphocytes, the so-called T helper cells. These cells are able to activate B cells upon engagement of their TCR. The activation of the appropriate B cell results in the production of antibodies and therefore in an activation of the humoral immune system. This connection is also evident by looking at the sources of antigenic peptides presented by the two different sets of MHC molecules. Whereas class I molecules primarily present endogenous peptide fragments, class II molecules bind peptides derived from proteins that were taken up into the cell by endocytosis. Since TAP is only involved in the class I presentation pathway, we will focus only on this pathway in the following sections.

1.2. Antigen processing and presentation by MHC class I molecules

In the class I MHC processing pathway, antigenic peptides derived from cellular proteins are presented to the TCR of $CD8^+$ T lymphocytes (Figure 1). In contrast to class II MHC molecules, which in principle connect the cellular and humoral immune system, class I MHC molecules are the tools used by the immune system to actively monitor events inside the cell. The engagement of the TCR by the MHC peptide complex signals the immune system to destroy this particular cell. Therefore, the class I MHC function enables the immune system to detect mutations that might result in cancer or cell malfunction as well as infiltration by pathogens. This essential protection can only be maintained if a constant formation of class I MHC peptide complex is ensured. Peptide fragments are generated mainly in the cytosol by a ubiquitous degradation machinery, the proteasome (for review see ref. [15]). This threonine protease generates protein fragments with a length preference of eight to thirteen amino acids but shows only little sequence specificity.[16] However, loading and class I MHC peptide complex formation take place in the endoplasmatic reticulum (ER), while most of the antigenic peptides are generated in the cytosol. Consequently, the membrane separating cytosol and ER represents an impermeable barrier that uncouples the generation of peptides from the loading reaction. Without further transport systems, the ER membrane would abolish any class I MHC mediated immune surveillance simply because this membrane prevents loading of peptides onto class I MHC molecules.

The 20S proteasome is the core of an ATP-dependent proteolytic machinery (for recent reviews see refs. [17, 18]). The "cylinder-shaped" core ($M_r \approx 700$ kDa) contains 28 subunits and is found in eukaryotes, prokaryotes, and archea. The recently accomplished X-ray structure analyses of the proteasomes from *Thermoplasma acidophilum*[19] and yeast[20] reveal an overall barrel architecture. The 28 subunits are arranged in four stacks with seven subunits per stack. β-Subunits form the two inner rings, whereas the α-subunits form the two outer rings. In mammals two subunits of the proteasome, termed LMP2 and LMP7, are encoded in the class II MHC locus.[21–23] This genetic organization led to speculations about a potential involvement of the proteasome in antigen processing and generation of peptide epitopes. The incorporation of these two subunits is inducible by interferon γ (IFN-γ), a common messenger molecule of the immune system. In addition, a third IFN-γ-inducible subunit, MECL1, has been identified, which is not encoded in the class II MHC locus.[24, 25] Over the years it has been demonstrated that the proteasome serves a twofold purpose. On the one hand, its proteolytic activity generates peptides which are degraded further by other proteases and subsequently metabolized to yield amino acids. On the other hand, IFN-γ-stimulated exchange of the "housekeeping" β-subunits for the subunits encoded in the class II MHC locus (LMP2, LMP7) and MECL1 creates "immunoproteasomes".[26] Evidence has been presented that incorporation of these subunits shifts the cleavage pattern of the "immunoproteasome" toward more tryptic and chymotryptic specificity.[27] This shift may enlarge the repertoire of potential antigenic peptides, but the exact mechanism of this switch as well as the general cleavage mechanism and specificity are still unknown.

As already pointed out, the generation of antigenic peptides in the cytosol requires an active transport system that shuttles peptides across the membrane of the ER (Figure 1). The transporter associated with antigen processing (TAP) performs this critical task. It is a heterodimeric transmembrane protein with an apparent molecular weight of approximately 150 kDa. This protein belongs to the family of ABC (ATP-binding cassette) transporters (see Section 2). TAP transports antigenic peptides in an ATP-dependent manner from the cytosol into the lumen of the ER in which the peptides are loaded onto class I MHC molecules. This ensures that the whole process does not rely on simple, passive diffusion of a charged species over a lipid bilayer. However, the proteasome is not the only protease present in the cytosol. In addition, the concentration of free peptides in the cytosol as well as in the lumen of the ER is extremely low. From a chemical and kinetic point of view, such a three-membered system consisting of a peptide-generating protease, the proteasome, a membrane-located transporter, TAP, and the accepting protein, class I MHC, would result in a very low transport efficiency under the conditions encountered in a cell. In addition, simply looking at the thermodynamic equilibrium constant of class I MHC peptide complexes highlights the problem even more. An equilibrium constant in the μM range reflects the fact that MHC molecules are by no means a trap for antigenic peptides. Simply relying on diffusion, competition between transporter binding and action of other proteases in the cytosol as well as collision as the only event leading to loading of class I MHC molecules cannot guarantee an effective antigen processing. Thus for complete and efficient unidirectional TAP-mediated transport of antigenic peptides from the cytosol into the lumen of the ER and for fast and efficient binding of the peptides to class I MHC molecules, accessory molecules are required.

1.3. TAP in concert with cofactors in loading of MHC class I

Little is known about factors involved in the process of transferring antigenic peptides from the proteasome to TAP in the cytosol. Some results indicate that the proteasome or

subfractions of ER-associated proteasome and TAP are in direct physical contact.[28] Consequently, peptides may not be released from the proteasome and may not diffuse freely within the cytosol. Rather, an unidirectional transport mechanism forwards peptides directly from their origin of generation to the transport machinery TAP. There is also evidence for the involvement of heat shock proteins in guiding peptides from the proteasome to TAP.[29] Although these hypotheses are appealing, they are still very much debated.

More is known about the involvement of proteins that are located on the luminal side of the ER. The assembly of class I MHC molecules is initiated by the "translocon ribosome" which shuttles the heavy chain of class I MHC molecules into the lumen of the ER (Figure 2). In the first step, the ER-resident chaperone calnexin binds to MHC heavy chain and stabilizes it.[30] The thiol-dependent reductase ERp57 assists in the correct formation of disulfide bonds within the heavy chains by forming a transient complex with class I MHC heavy chain and calnexin.[31–33] Subsequently, β_2-microglobulin (β_2m) enters the complex and nascent class I MHC molecules are assembled. At this stage the protein calreticulin, located in the lumen of the ER, enters the complex and displaces calnexin.[34] Calnexin and calreticulin act as chaperones for glycoproteins within the ER. Despite this common function, calreticulin binds preferably to assembled class I heavy chain – β_2m dimers. In addition, calreticulin may act in an editing cycle which retains immature class I MHC molecules within the ER and thereby prevents export and transport to the cell surface (Figure 2). Finally, the assembly process cumulates in the formation of a macromolecular "transport and loading complex" that consists of class I MHC molecules, TAP, and tapasin (TAP-associated glycoprotein). It was shown that approximately four class I MHC and four tapasin molecules bind to TAP.[35] The ER-resident protein, tapasin, plays an essential role in forwarding peptides from TAP to class I MHC molecules.[36] Within the "loading complex", tapasin serves a twofold purpose. Tapasin lacking the transmembrane domain and the cytosolic tail is still able to promote surface expression of class I MHC molecules but does not participate in the "loading complex".[37] In addition, tapasin increases the level of TAP, which simply results in a higher amount of peptides being transported across the ER membrane.[37] Subsequently, antigenic peptides are transferred from TAP to the MHC molecule. The physical contact between TAP and MHC dramatically increases the local peptide concentration. After forwarding peptides to the MHC molecules, the ternary complex falls apart, and class I MHC peptide complexes are transported to the cell surface.[38, 39] With this complex network of proteins, a guided and extremely efficient loading of antigenic peptides, generated in the cytosol by the proteasome, via TAP onto class I MHC molecules is ensured. Nevertheless, it is very likely that even more proteins will be identified that are involved in the assembly of class I MHC molecules and subsequent loading of peptides onto class I MHC molecules.

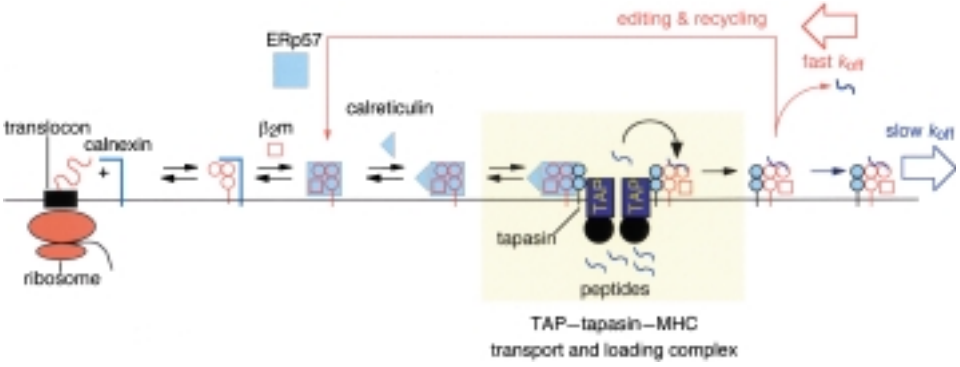

Figure 2. *Schematic illustration of the class I MHC assembly pathway within the ER. The TAP – tapasin – MHC complex, the so-called loading complex, plays a central role in the generation of matured class I MHC peptide complexes. Details of this spatially and temporally organized transport and loading complex are given in the text.*

2. The family of ABC transporters

Since the first description of an ABC transporter in the early 1980s,[40] the number of proteins identified as belonging to this family has increased exponentially with the availability of data from genome projects. Nowadays, ABC transporters are believed to form the largest family of paralogous proteins in several organisms. The designation ABC transporters recognizes a highly conserved ATP-binding cassette,[41] which is the most characteristic feature of this protein family. Based on sequence comparisons, 2% of all gene products in the Gram-negative and Gram-positive bacteria *Escherichia coli* and *Bacillus subtilis*, respectively, are ABC transporters, 28 ABC transporters are encoded in the yeast genome, while between 70 and 100 of these proteins may be encoded in the human genome. The functions range from transport of heavy metals, such as arsenic, uptake of nutrients, such as sugars and amino acids, to peptide and protein export (for review see refs. [42 – 44]). In addition, cystic fibrosis, which is the most common deadly inherited disease in Caucasians, is related to a mutation of an ABC transporter, the cystic fibrosis transmembrane conductance regulator (CFTR) protein. Another ABC protein, the multidrug resistance protein (MDR1), also called P glycoprotein (Pgp), is of high medical importance and constitutes one of the major limitations in chemotherapy. Although performing different functions, all of these proteins including TAP share a common architecture that forms the structural basis for this family of membrane-spanning transport proteins (Figure 3). In mammalian organisms CFTR and MDR1 are probably the best-characterized proteins of this family. Therefore, we will first summarize some of the general structural and mechanistic aspects of ABC transporters, with an emphasis on CFTR and MDR1. After this introduction to ABC transporters in mammals, we will focus on TAP, with an emphasis on mechanistic and structural aspects of this important protein.

2.1. Architecture of ABC transporters

All ABC transporters described so far show a four-domain organization and utilize ATP as the source of energy (Figure 3). The basic blueprint consists of two transmembrane domains (TMDs), which are thought to perform the transport function, and two nucleotide-binding domains (NBDs) providing the energy for transport. The four domains are arranged in all possible combinations. In Figure 3 prominent examples of the

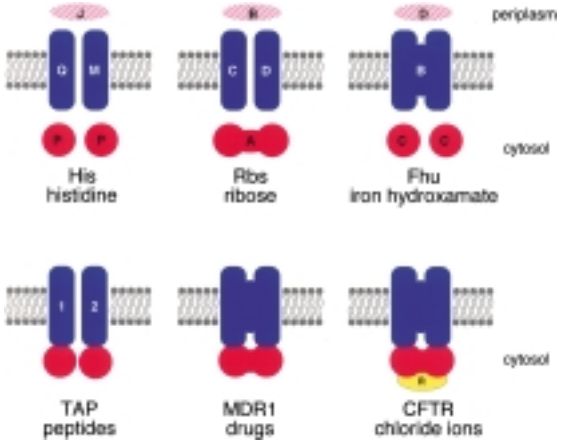

Figure 3. *Structural organization of ABC transporters. TMDs are shown in blue, NBDs in red. Depicted are selected, characteristic examples for the diverse architecture of ABC transporters from bacteria (top row) and mammals (bottom row). Additionally, substrates are listed and the orientations of the transporter within the membrane are indicated. Bacterial ABC transporters work in concert with a periplasmic substrate-binding protein (purple stripes). Single letters or numbers indicate individual subunits of ABC transporters. In the case of CFTR, R denotes the regulatory domain (yellow) which is unique to CFTR.*

family of ABC transporters are summarized schematically to demonstrate the diversity of these fascinating membrane proteins. In eukaryotes the four domains are generally found on a single polypeptide chain. In the case of TAP the active transporter is composed of two subunits, TAP1 and TAP2, which contain one TMD and one NBD each. Although the function and general organization of ABC transporters are known, it is still unclear whether particular proteins form a channel, a pore, or function in a ligand-flipping fashion.

2.2. Cellular functions and mechanistic aspects of ABC transporters

Probably the two best-known and -characterized members of the family of mammalian ABC transporters are CFTR and MDR1. CFTR is a plasma-membrane-spanning, protein kinase A (PKA) dependent chloride channel[45] from the apical site of epithelia cells[46] and consists of 1480 amino acids.[47] It is known that deletion of a single amino acid (ΔF508) results in a lowered chloride ion conductance. This deletion is the most common mutation in cystic fibrosis.[48] It leads to the production of highly viscous mucus, a commonly encountered situation in cystic fibrosis. Additionally, it has been speculated that CFTR regulates "outwardly rectifying chloride channels".[49] It has to be stressed that the movement of chloride ions across the plasma membrane occurs along an electrochemical gradient in a channel-like fashion and is not simply an ATP-driven transport. Transporters and pumps couple energy (e.g., generated from ATP hydrolysis) stoichiometrically to the active transport of the substrate. Channels, on the other hand, normally need no energy. In addition, channels are diffusion-limited, and the turnover number is several orders of magnitude larger than that of a transporter. Thus, channels simply facilitate equilibration of the substrate in response to concentration or electrochemical gradients. In contrast, transporters utilize energy to transport substrates against a gradient. This distinction has important mechanistic consequences for CFTR. Isolation and reconstitution of purified CFTR in artificial membranes demonstrated that CFTR forms a channel.[50] It has been shown that ATP hydrolysis does not provide the energy for chloride ion transport but maintains channel conductance.[51] Subsequently, it was proposed that ATP hydrolysis plays a role in opening and closing of the channel pore.[52, 53] Further studies demonstrated that both NBDs are nonequivalent and furthermore indicated distinct functions and a subtle regulation mechanism of gating by the NBDs.[52, 54] Moreover, Kopito and co-workers[55] observed two open states of CFTR. The transition between both open states violates the principle of microscopic reversibility and necessitates an external source of energy. This energy is provided by ATP hydrolysis and indicates that CFTR represents an unusual ligand-gated ion channel. NBD1 primes the channel by ATP hydrolysis and converts the protein from an inactive into an active but still closed conformation. Subsequent binding of ATP to NBD2 switches the conformation of CFTR to the open state (O_1). Hydrolysis of ATP bound to NBD2 creates another open conformation (O_2). Dissociation of ADP or P_i generates the active but closed conformation. Conversion into the closed, inactive state occurs when ADP or P_i dissociate from NBD1.[55]

Human MDR1 is a single polypeptide chain consisting of 1280 amino acids (150 – 170 kDa). Common to all ABC transporters are two hydrophobic transmembrane domains and two hydrophilic ATP-binding domains.[56] This glycoprotein forms an energy-dependent drug extrusion system that is one of the major causes of multidrug resistance in tumor cells.[57] Consequently, most chemotherapeutics fail for a large number of human cancers including colon, kidney, and breast carcinomas as well as leukemia. In contrast to CFTR, no cellular function has been determined so far for this clinically relevant membrane protein. Except for the observation that MDR proteins might act as a lipid translocase,[58] the cellular function is still very much under debate. MDR1, as opposed to CFTR, is a classic transporter that utilizes ATP hydrolysis in a stoichiometric fashion to pump a vast variety of drugs against a concentration gradient. MDR1 substrates include anticancer drugs such as vinca alkaloids, anthracyclines, antibiotics, taxol and its derivatives, or cytotoxic agents such as antimicrotubule drugs (colchicine), protein synthesis inhibitors (puromycin), DNA intercalators (ethidium bromide), or toxic peptides (gramicidin D, valinomycin), and fluorescent dyes such as rhodamine 123.[56, 59] All these substrates have only their hydrophobicity in common. Additionally, it has been observed that MDR1 achieves both reducing drug influx into the cytosol and increasing drug efflux from the

cytosol.[60, 61] Taking all these data together, a hydrophobic "vacuum cleaner" model has been proposed.[56] Here the substrates of MDR1 are recognized and bound from the inner leaflet of the plasma membrane and expelled.[62] Due to their lipophilicity, drugs that have already entered the cytosol will repartition between the aqueous cytosol and the lipid bilayer of the plasma membrane (Figure 4). Consequently, recognition, binding, and expulsion of these substrates will perturb the equilibrium of membrane partition and additional drugs will re-enter the plasma membrane from the cytosol. Based on this

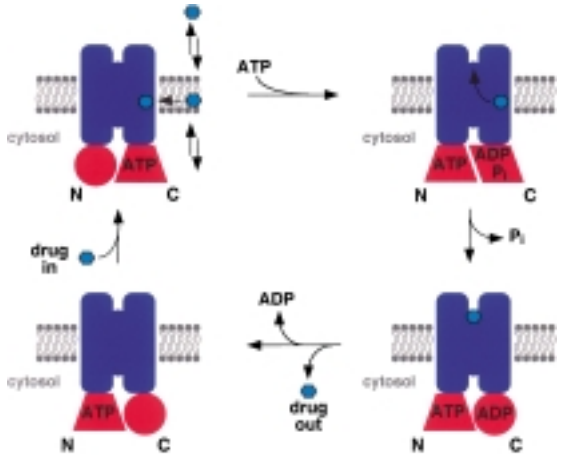

Figure 4. *Catalytic cycle of MDR1. The model was adapted from ref. [71]. Further details are given in the text.*

proposed mode of action, MDR1 both hinders drugs from entering the cytosol and extrudes them (Figure 4). While it is accepted that MDR1 utilizes ATP hydrolysis to energize the extrusion of drugs, it is still unclear why MDR1 shows a high basal ATPase activity. This might point to the cellular function of MDR1, for example the translocation of lipophilic membrane components.[58] Nevertheless, the ATPase activity is stimulated up to 10-fold in the presence of substrates with a maximum turnover number of 10 s^{-1}.[63] In contrast to other ATPases such as myosin or F_0F_1-ATPase, which bind ATP with high apparent affinity, MDR shows low affinity for ATP ($K_M \approx 10^{-3} - 10^{-5}$ M). The absence of any phosphorylated enzyme intermediate distinguishes MDR1 from P-type ATPases such as the (Na^+,K^+)-ATPase.[64] These findings led to the conclusion that ATP hydrolysis creates a conformational state of MDR1 with a high chemical potential. Relaxation from this state generates the energy necessary to transport drugs. Despite the high basal ATPase activity which discriminates MDR1 from P-, V-, and F-type ATPases, ATP hydrolysis and drug transport seem to be coupled. The stoichiometric ratio of hydrolyzed ATP to transported substrate molecule is nearly 1:1, 0.83 ATP per transported rhodamine 123[62] and 0.5 to 0.8 ATP per transported valinomycin.[65] These observations raised the question why two NBDs are present in MDR1. Both NBDs bind ATP with similar affinity[63, 66] and have the capacity to hydrolyze ATP.[67] However, it was shown that both NBDs possess a positive catalytic cooperativity.[68, 69] Inhibition of only one of the two ATP-binding sites abolished drug transport completely.[70] In summary, a working model (Figure 4) has evolved which shows striking similarities to the mechanistic cycle of CFTR. ATP is bound to one of the NBDs (C site), and substrate binds at the cytosolic side of both TMDs. Binding of ATP to the second NBD (N site) results in hydrolysis of ATP in the C site and formation of a conformational state with high chemical potential.[63] Relaxation of this state to a state with lower potential results in release of P_i from the C site and transport of the substrate to the extracellular site of the transporter. Release of the drug and dissociation of ADP from the C site results in reformation of the substrate-accessible ground state. The only difference is that N and C sites are now inverted[71] (Figure 4). Such a scenario implies that both NBDs are able to act symmetrically. However, it is presently not clear whether both NBDs work symmetrically or whether one NBD promotes the formation of the ground state of the ABC transporter.

2.3. Nucleotide-binding domain of ABC transporters

Irrespective of the organism or the cellular function, all ABC transporters are categorized based on a consensus sequence of the ATP-binding domain and the four-domain organization. As will be discussed later (see Section 2.4), both TMDs form the substrate-binding site(s) and consequently show the lowest sequence homology. On the other hand, the NBDs provide the required energy and have a high degree of sequence identity.

In the absence of any high-resolution structural information, establishing the architecture of the TMDs and NBDs relied mainly on hydrophobicity analyses, biochemical and mutational studies, and homologies to other proteins with known three-dimensional structures. The NBDs of ABC transporters share a sequence identity of approximately 30 % over a stretch of 200 amino acids. Within this stretch, three conserved motifs emerged from sequence alignments. The first stretch, the so-called Walker A motif,[41] is a glycine-rich loop ("phosphate-binding loop", P loop) with the consensus sequence GXXXXGKS/T (Figure 5 B). Based on the three-dimensional structures of adenylate kinase (AK),[72] Ras p21,[73] F_1F_0-ATPase,[74] elongation factor Tu (EF-Tu),[75] and the recently solved X-ray structure of the NBD of histidine permease (HisP),[76] this loop coordinates the phosphate groups of ATP (Figure 5 A). The second consensus sequence is the Walker B motif,[41] a stretch of four hydrophobic amino acids and an aspartate or glutamate (Figure 5 B). This stretch is also observed in AK, Ras p21, F_1F_0-ATPase, and EF-Tu; it participates in phosphate binding through a conserved water molecule. The third consensus sequence motif, the C loop (GGQR/KQ), is found in all ABC transporters and G proteins.[77, 78] The C loop is thought to be central to connecting ATP hydrolysis with function.[78–80] Based on the sequence homology of the NBDs to other ATP- and GTP-binding proteins, various models have been proposed.[78, 80–82] Depending on the lead structure, different structural models for the NBD were obtained. Surprisingly, the recently solved structure of HisP[76] revealed a complete new protein fold with only limited similarities to F_1F_0-ATPase[74] and RecA.[83] The overall shape of HisP is that of an "L"; the ATP-binding pocket is composed of the Walker A and B motifs and is

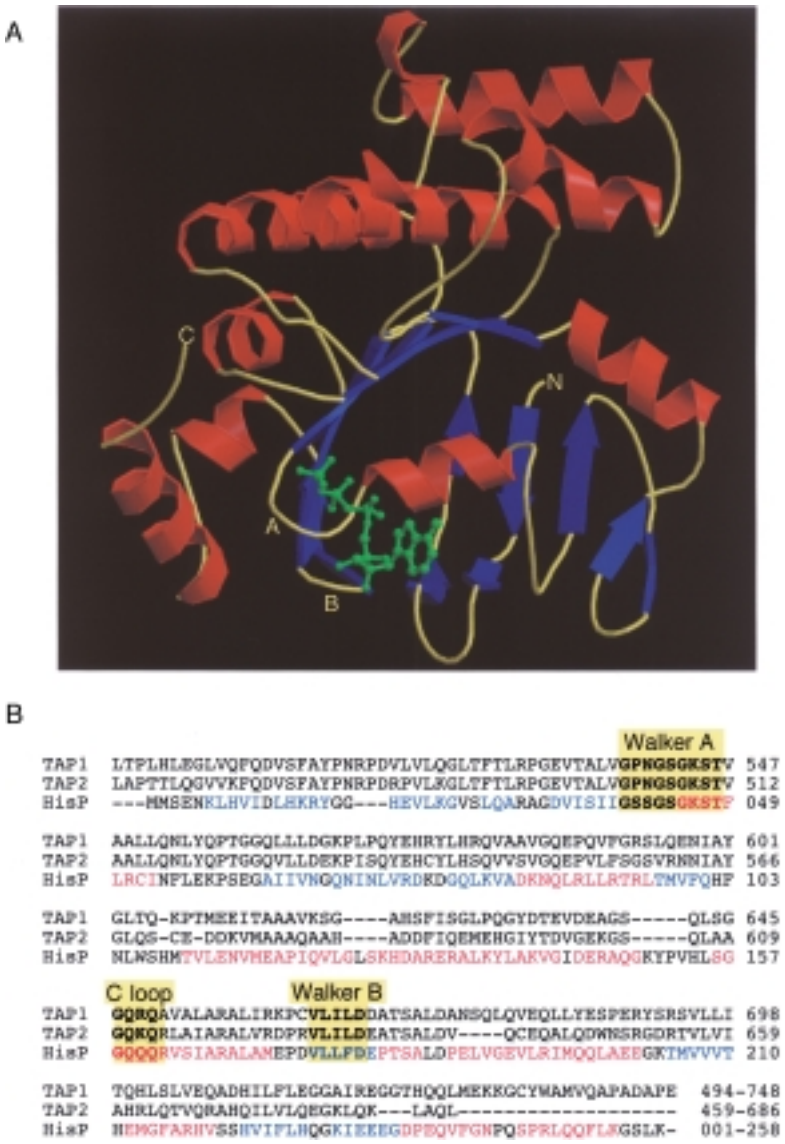

Figure 5. *A: High-resolution structure of HisP,[76] the ATP-binding domain of the histidine permease complex. Coordinates of HisP were kindly provided by Dr. H.-S. Kim, University of California at Berkeley, prior to release from the Brookhaven protein data bank.[76] Helices are drawn in red, sheets in blue, loops and turns in yellow, and ATP in green. B: Sequence and secondary-structure alignment of the NBDs of TAP1 and TAP2 on the sequence of HisP. The secondary-structure alignment is based on the structure of HisP. Residues shown in blue indicate* β *sheets and residues in red* α*-helical regions. Secondary-structure elements are only indicated for HisP. Sequences were obtained from the Swiss Genbank. Alignments were performed using the ClustalW service at the European Bioinformatics Institute.[205]*

located at the end of one arm of the L (Figure 5 A). The C loop is located at the opposite end of the other arm. Both arms are spanned by a six-stranded β sheet, which is surrounded by an α/β-type structure on one side and a domain of mostly α helices on the other side. The ATP-binding site is composed of residues belonging to the Walker A and B motifs. Because the crystals were obtained in the presence of ATP but in the absence of Mg^{2+}, any detailed interpretation of the precise arrangements within the ATP-binding pocket will have to wait until the structure of the $Mg^{2+}\cdot$ATP complex has been solved. Nevertheless, the recently reported structure of RbsA,[84] the ATP-binding domain of the ribose import system, demonstrated a high degree of similarity to the HisP structure. Whether or not this structural information will be valid for other NBDs, especially those from export systems or mammalian sources, remains to be seen.

2.4. The transmembrane domain of ABC transporters: substrate recognition and translocation channel

Based on the sequence homology one can expect a high degree of structural similarity among NBDs of ABC transporters. In contrast, the TMDs show less sequence homology. All of the experimental data so far indicate that the TMDs form the binding and translocation site of ABC transporters. Early data obtained for bacterial ABC transporters predicted six transmembrane helices per TMD.[85] Over the years, the six-helix motif was confirmed experimentally for other ABC transporters including CFTR and MDR1.[56, 86–90] Nevertheless, exceptions from the six-helix paradigm were observed. For example, hydrophobicity analysis predicted eight transmembrane helices for the maltose transporter MalF.[91] Other examples include the histidine transporters HisQ and HisM,[40] the manganese transporter MntB,[92] MDR1,[93] and TAP1.[94] Further analyses provided strong evidence that the two additional transmembrane helices of MalF are not necessary for the in vivo function of the transporter.[95] Thus, the six-helix organization is very likely the core motif of ABC transporters, whereas different numbers of transmembrane helices can be regarded simply as an extension.

The general architecture of ABC transporters consists of a six-helix TMD, NBD1, the second six-helix TMD, and NBD2. Thus, adopting the same topology, the differences in amino acid composition of the TMDs create various overlapping binding sites for a large spectrum of ligands and transported substrates. Consequently, different functions can be performed. On the other hand, the sequence homology of the NBDs indicates a similar or even identical fold that serves the purpose of providing energy and coupling ATP hydrolysis to function. In the case of MDR1, photoaffinity labeling indicated that more than one drug-binding site exists.[96] In addition, amino acids of both TMDs participate in substrate binding.[97, 98] This picture is consistent with the low-resolution structure of MDR1.[99] MDR1 has the overall shape of a cylinder, with a diameter of approximately 10 nm, a maximum height of 8 nm, and a central cavity of about 5 nm in diameter. Surprisingly, the cytoplasmic site of the central pore seems to be closed. Both NBDs presumably achieve this closure, which indicates a strong "communication" between the single domains. ATP-induced changes of the tertiary structure have been also observed by IR

spectroscopy.[100] In summary, a picture is emerging in which ATP hydrolysis and/or substrate binding induces major conformational rearrangements of the protein. These changes are transmitted to the TMDs that form one or more ligand-binding sites.

3. Structural aspects of TAP

TAP is a heterodimeric transmembrane protein located in the membranes of the ER and cis-Golgi.[101] The central role of TAP in class-I-mediated immune response was first observed in cell lines lacking the TAP genes.[102] Transfection of one or both genes into these cell lines restored the class I MHC mediated presentation of antigenic peptides.[103, 104] These transfection studies as well as heterologous expression in insect cells and yeast demonstrated that both TAP subunits were necessary for transport function, indicating that a heterodimeric complex composed of TAP1 and TAP2 is involved in antigen processing.[105–108] The genes of TAP1 and TAP2 are located within the class II MHC locus in close proximity of two proteasomal subunits, LMP2 and LMP7 (see Section 1.2).[109] Both TAP genes are made up of eleven exons. Eight of these have the same size, and all exon/intron boundaries are identical.[110] As derived from the DNA sequence, human TAP1 is composed of 748 amino acids (calculated molecular weight: 81 kDa) and TAP2 of 686 amino acids (calculated molecular weight 75 kDa). Cloning of the cDNAs and sequence analyses revealed a strong homology of TAP to the family of ABC transporters. In addition, sequence comparisons with other ABC transporter revealed a close homology to MDR1. Isolated microsomes and semipermeabilized cells were employed to finally prove that peptide transport from the cytoplasm into the ER lumen was TAP- and ATP-dependent.[111–113] Colocalization experiments and immunogold labeling demonstrated that the cellular location of TAP was restricted to the ER membrane and the cisternae of the cis-Golgi apparatus.[101] However, as no ER retention signal was determined for TAP, a yet unknown mechanism seems to retard TAP in these subcellular compartments. Accessibility of domain-specific antibodies was used to investigate the in vivo orientation of TAP.[114, 115] Based on these and other studies, it is now commonly accepted that the NBD domains of both TAP subunits face the cytosol. In vivo studies showed that both subunits associate very rapidly to form the functional transporter.[116] In contrast to MDR1 and CFTR, only a very small subpopulation of TAP appears to be glycosylated although three putative glycosylation sites exist.[116] Thus, it seems likely that the portion of N-glycosylated TAP represents misfolded protein.

3.1. Membrane topology of TAP

The sequence homology between full-length TAP1 and TAP2 is around 35%. However, the sequence homology for the NBD is around 60%, as expected for a member of the family of ABC transporters, whereas the homology drops to only 30% for the TMDs.[117]

In prokaryotic organisms membrane proteins are oriented within a membrane on the basis of the "positive inside rule".[118] In the case of eukaryotic membrane proteins, the charge difference of the first 15 residues flanking the transmembrane region determines its orientation. Based on this observation the more positive portion will face the cytosol.[119] Further elaboration of this observation showed that clusters of positively charged amino acids (arginine and lysine) are found at the cytosolic site of the membrane for polar stretches connecting two transmembrane helices if the loop is shorter than 60 amino acids.[120] Depending on the algorithm used, six to ten transmembrane helices were predicted for TAP1 and TAP2.[94, 121–124] In addition, sequence comparisons with other ABC transporters demonstrate that the first 175 N-terminal residues of TAP1 and the first 140 N-terminal residues of TAP2 have no counterparts (Figure 6). In these so-called N domains three to four transmembrane helices are predicted.[124] Following the "six-helix dogma", the helical transmembrane "core motif" of TAP1 and

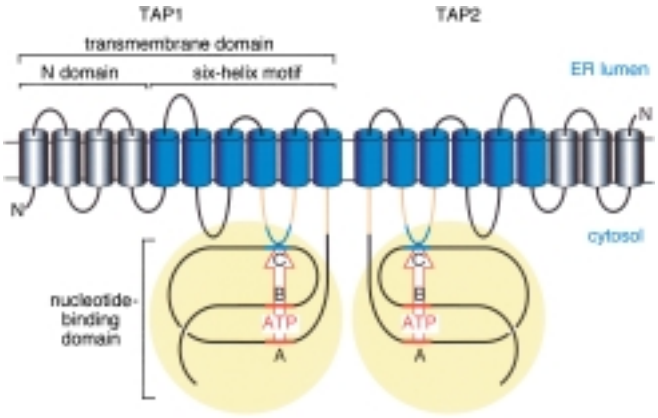

Figure 6. *Topology model of TAP. Putative transmembrane helices of the N domains[124] are shown in gray, the transmembrane helices forming the six-helix motif of ABC transporters are shown in blue. The peptide-binding region is indicated in orange. The triphosphate moiety of ATP is bound through interactions to amino acids of the Walker A and B motifs. It is assumed that hydrolysis of ATP results in conformational changes within the NBD, which are transmitted to the TMDs by the C loop (arrows from A to C).*

TAP2 starts around positions 175 and 140, respectively. Accordingly, these six transmembrane helices can be labeled TM1 to TM6 on the basis of the sequence alignment and the homology to P glycoprotein (Figure 6). The remaining N domains are thought to be an extension that was acquired during evolution and might be necessary for the function and specialization of the complex. The topology predictions demonstrated that the TAP complex is highly asymmetric with respect to both membrane sites. In addition to the NBDs, there are large cytoplasmic loops, but only a small portion of the transmembrane helices containing loops reach out into the ER lumen.

3.2. The nucleotide-binding domains of TAP

The recently solved X-ray structure of HisP, the NBD of the histidine transport system of *E. coli*, revealed a new protein fold.[76] Despite the presence of Walker A and B motifs[41] commonly found in ATPases,[125] the additional C loop, specific for ABC transporters as well as the new fold observed in HisP,

indicates that NBDs of ABC transporters might form a distinct family of proteins. Whether or not this fold will be generally found in all ABC transporters cannot be answered yet. However, it is tempting to speculate that this "ABC transporter fold" is used to enable a specific crosstalk between the "motor domain" (NBD) and the substrate-binding and "transporter domain" (TMD). Maybe NBDs are tailored not only to generate the necessary energy but also to transmit the signal of hydrolysis and induce conformational changes that allow subsequent transport to occur. But more importantly, structural information is only available for the ATP-bound state of HisP in the absence of Mg^{2+}. One has to expect conformational changes with respect to the structures of the empty, $Mg^{2+} \cdot$ ATP, ADP – vanadate-, or the ADP-bound forms of the NBD. To answer these questions one has to wait until the structures of these different functional states have been solved. Nevertheless, we have used the structure of HisP to model the NBDs of TAP1 and TAP2. Sequence alignment (Figure 5) and superposition of human TAP1 and TAP2 onto the structure of HisP indicate that the only major differences in sequence occur within the loops of HisP. The overall fold remained the same, and differences between template and model occur only in loop regions. Interestingly, the loop preceding the C loop is shorter in TAP1 or TAP2 than in HisP. Whether or not this indicates a subtle fine-tuning in the communication pathway between NBD and TMD remains unclear.

Despite this speculation it was shown that peptide translocation comprises at least two mechanistic steps.[114] The first step, peptide binding, does not require nucleotide triphosphates, whereas the second step, peptide transport, needs to be energized by the hydrolysis of nucleotide triphosphates. It is interesting to note that isolated expressed NBDs of TAP are able to bind various nucleotide triphosphates independently of one another, but they do not hydrolyze ATP.[126, 127] This observation implies that hydrolysis of ATP is coupled to signals arising from the TMDs of TAP. Alternatively, this observation might indicate that nucleotide triphosphate binding induces a conformational change within the protein,[128, 129] which subsequently engages the ATPase activity of the NBD[130] similar to a feedback mechanism.

3.3. Structure – function relationships

Studies on peptide binding and cross-linking to TAP have revealed that a separate peptide-binding step precedes the actual transport step.[114, 131–133] Moreover, competition experiments suggested that only one single peptide-binding site exists.[114, 132, 133] Modified peptides with a photoaffinity label in combination with epitope-specific antibodies were employed to map the peptide-binding site in more detail.[121, 134] Despite their difference in sequence, all peptides labeled both TAP subunits. However, the ratio of both TAP subunits labeled varied very much depending on the actual position of the label within the peptide. In case of TAP1, labeling was observed within two regions. One (amino acids 376 – 391) is located in the cytoplasmic loop connecting TM4 and TM5 (Figure 6). The nomenclature is based on the standard nomenclature for ABC transporters neglecting putative helices of the N domain. The second region (amino acids 475 – 487) is located in the cytoplasmic loop connecting TM6 and the NBD. Thus, regions of TAP1 closest to the NBD were labeled. For TAP2, a mirrorlike situation was observed.[121] Based on these data, the authors proposed that ATP hydrolysis induces a conformational change on TAP that is transmitted to the TMD. On a molecular level, these data conclusively explain why only a heterodimeric TAP complex can bind and subsequently transport peptides. Only the correct quaternary structure of the complex ensures the formation of a functional binding site for peptides and thus their translocation. Based on these studies, the influence of polymorphic residues on peptide specificity and transport was analyzed.[122, 135] New polymorphic clusters in rat TAP2 were determined that influence the peptide specificity of TAP. In addition to the loop connecting TM4 and TM5, a region between TM2 and TM3 (amino acids 183 to 185) was identified that determines peptide specificity.

These results indicate a cooperative interaction between different regions of the protein. Consequently, a high degree of communication between the loops as well as the NBD has to be expected. The close proximity between NBDs and peptide-binding site implies that one site can sense even the slightest conformational changes at the other site and vice versa. Whether both NBDs are equivalent or different—as has been shown for MDR1 and CFTR—is still an open question. It is also interesting to note that so far all residues forming the ligand-binding site are located close to the polar headgroups of the lipid bilayer. Consequently, it is tempting to speculate that the transmembrane helices solely form a pore or scaffold that translocates peptides upon receiving a signal which is triggered by the crosstalk between the peptide-binding site and NBDs. The molecular mechanism of TAP therefore is distinctly different from the proposed mechanism of MDR1. For MDR1, at least two substrate-binding sites have been identified, which are located within the lipid bilayer spanning part of TAP. Nevertheless, the data so far indicate a strong communication at least in the cytoplasmic part of the protein. How this communication is achieved on a molecular level has to await further structural and mechanistic studies. But one has to keep in mind that the energetization of the transport process by ATP hydrolysis is the prerequisite for TAP function. Mutation of the NBD near the Walker B motif abolished the peptide transport function of TAP although the protein was expressed on a normal level.[136] This demonstrates dramatically how important the yet unknown mechanisms of communication between the domains of TAP are and how subtle the molecular regulation of these individual steps is.

4. Affinity, specificity, and diversity of TAP

Human class I MHC molecules are separated into three allelic series of peptide-binding proteins. Due to an extensive genetic polymorphism of these three alleles, a repertoire of class I MHC molecules exists in every human that is able to present, at least in theory, almost every peptide sequence of eight to ten amino acids to the TCR of T lymphocytes. As for TAP, the central question was: How do class I MHC molecules achieve this task?

The high-resolution X-ray structures of class I MHC molecules[137–141] and pool-sequencing of peptides eluted from class I MHC molecules[142–145] have deciphered the principles of affinity and specificity. Class I MHC molecules are heterodimers consisting of a membrane-anchored heavy chain and noncovalently associated β_2m (Figure 7). The MHC heavy chain is composed of the extracellular domains α1, α2, and α3. α3 and β_2m adopt a standard immunoglobulin fold. Each of these domains consists of a sandwich formed by a three-stranded and a four-stranded β sheet that are packed against each other. The peptide-binding groove is built up by domains α1 and α2, which form an "intrachain dimer".[137] Each domain contributes four β strands forming an eight-stranded antiparallel β sheet, which builds up the floor of the peptide-binding groove. Additionally, two long α helices flank the floor of the groove. The resulting groove, which is approximately 30 Å long and 12 Å wide, is tapered and blocked completely by bulky aromatic amino acids at both ends. The structures provided a molecular framework as a basis to explain the impressive ability of MHC molecules to bind and present antigenic peptides to TCRs.[146] Out of the eight to ten amino acids that are bound by the class I MHC molecule only three positions are important for the specificity of the protein–ligand interaction (Figure 7). These positions are called anchor residues.[147] Consequently, only a minor part of the antigenic cargo participates in the selectivity of the complex formation between class I MHC molecule and peptide. Conserved pockets of the MHC molecule at both ends of the peptide-binding groove accommodate the amino (pocket A) and the carboxy termini (pocket F) of the peptide. The third position is a deep polymorphic pocket (pocket C), which provides structural complementarity to the main anchor residue of the peptide and determines the MHC-allele-specific peptide-binding pattern. Consequently, only a few MHC molecules are capable of binding a vast number of different peptides. The recognition principle is based mainly on rather unspecific interactions. Almost all hydrogen bonds are formed between the protein and the peptide backbone. The large number of van der Waals contacts is due to the deep burial of the peptide within the binding cleft. These unusual features promote promiscuous rather than specific interactions while still maintaining a high degree of affinity.[148] Does TAP bind peptides in a similar fashion? Do rather unspecific interactions determine the affinity? In the absence of structural information, alternative approaches were chosen to decipher the binding principle of TAP.

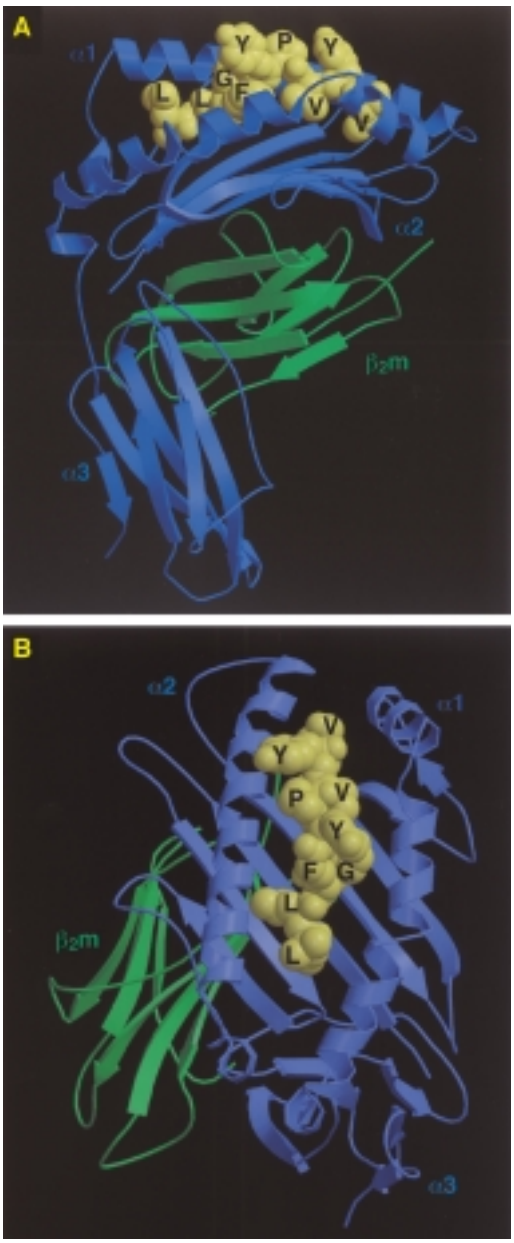

Figure 7. *High-resolution structure of a class I MHC molecule with a bound peptide. Side-view (A) and top-view (B) of HLA-A2 in complex with the peptide LLFGYPVYV derived from human T cell lymphotropic virus type 1 (HLTV-1) tax protein (PDB entry 1ao7).[206] The MHC heavy chain comprising subdomains α1, α2, and α3 are shown in blue, the β_2m subunit is shown in green, and the antigenic peptide in yellow. Peptide side chains ranging from position P3 (Phe) to position P8 (Tyr) are pointing upward and serve as a putative recognition surface of the TCR. Side chains that act as anchor residues (P1, P2, and P9 corresponding to residues Leu, Leu, and Val) are located in deep pockets of the MHC molecule and are responsible for specificity and stability of the complex.*

4.1. In vitro transport and binding assays for the investigation of the biological function of TAP

Functional studies of TAP are hampered by the amount of available protein and the stability of the protein in an artificial environment such as a detergent micelle or lipid vesicle. So far, only two reconstituted in vitro systems have been described.[149, 150] To establish an in vitro assay for peptide transport by TAP, microsomes[113] or semipermeabilized cells[111, 112] were employed. In the case of semipermeabilized cells, the plasma membrane is partially disrupted by the bacterial pore-forming toxin streptolysin O and peptides can be introduced directly into the cytosol (Figure 8). In the case of microsomes, cells are lysed and microsomal vesicles are isolated by sucrose gradient centrifugation. Transported peptides are trapped in the ER by binding to MHC class I molecules or, more conveniently, by glycosylation, which requires placing an N glycosylation con-

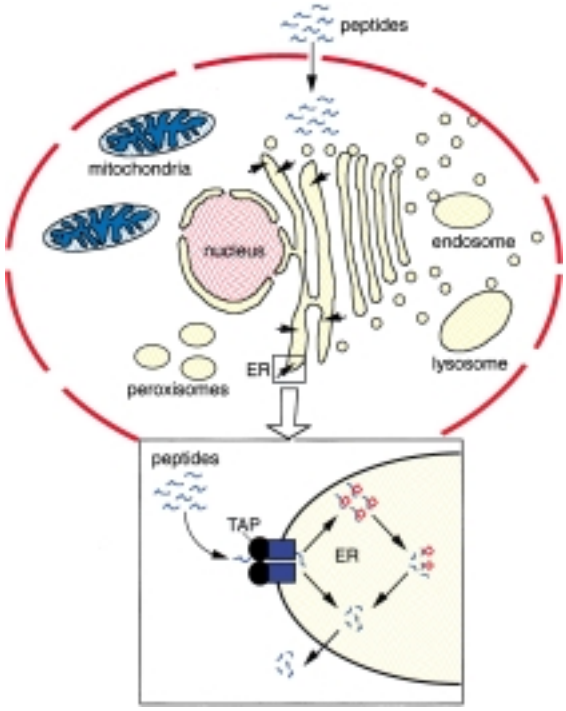

Figure 8. *Peptide transport and binding assays with semipermeabilized cells or~isolated microsomes. Peptides are transported in an ATP-dependent manner into the ER lumen. As indicated, side reactions such as trimming, degradation, or re-export of transported peptides by a yet unknown exporting machinery may occur and compete with the glycosylation of the reporter peptides or their subsequent loading to class I MHC molecules.*

sensus sequence (NXS/T) within the peptide. Glycosylated and thus translocated peptides are quantified by binding to a sugar-specific lectin.[112] The transport efficiency is reflected by the extent of glycosylation. However, there are still some problems that are summarized schematically in Figure 8. Side reactions of the peptides, such as trimming, transport back into the cytosol, or simply degradation, are severe drawbacks of such an assay. Thus, peptide glycosylation does not necessarily reflect differences in peptide transport or peptide affinity for TAP. Even competition experiments in the transport assay have to be interpreted very carefully. As it has not been established yet whether peptide binding, transport, or glycosylation is the rate-limiting step in these assays, no quantitative interpretation is possible. Even a comparison of different peptides and their transport characteristics has to be undertaken with great caution.

To simplify and reduce the number of unknowns, an assay was developed that analyzes only the step of peptide binding to TAP. The dissection of binding and transport of peptide is achieved by ATP depletion and low temperature.[114, 133] In combination with competition experiments, peptide affinity and the principles of substrate recognition and sequence specificity of TAP were extracted from these experiments. In contrast to the transport assay, which is a multimolecular reaction with its own kinetic parameters and specificity, the binding assay is a simple bimolecular reaction that can be described very accurately, at least in theory.

4.2. Length and sequence specificity of TAP

Confronted with the vast number of peptides that are presented by class I MHC molecules and therefore substrates for TAP, several research groups started to elucidate the molecular principles by which TAP selects its substrates. In a first set of experiments the length preference of TAP was investigated. The minimal length requirement for binding and/or transport is eight amino acids.[115, 151–153] Furthermore, free N and C termini are a strict requirement for transport.[115, 154] This points to a clear correlation with the length preferences of class I MHC molecules.[147] Interestingly, Koopmann et al. observed equal transport efficiencies for peptides of 8 – 12 amino acids,[153] whereas van Endert et al. determined equal binding affinities for peptides ranging from 8 to 16 amino acids in lenght.[114] However, no clear cutoff for the maximal length of transported peptides could be detected. Generally, an increased peptide length resulted in a decreased transport efficiency.[151–153] Interestingly, class I MHC molecules have been identified that possess the capability of binding peptides of up to 33 amino acids.[155] One could speculate that the transport ability of TAP for longer peptides[153] simply supplies such MHC molecules. On the other hand, it is very likely that longer peptides are trimmed in the ER, thereby enlarging the pool of antigenic determinants. There is experimental evidence that trimming of peptides in the cytosol and ER occurs.[156, 157]

The TAP genes are encoded in the class II MHC locus, and coding sequence polymorphism has been described in human, mouse, and rat. The observed variations in sequence comprise a series of dimorphisms at various positions along the polypeptide chain. Therefore, TAP alleles are defined in terms of the particular combinations of these dimorphisms. No functional relevance is associated with polymorphism of human and mouse TAP regarding peptide specificity.[115, 154, 158, 159] A remarkable exception is the rat TAP2 gene. The allelic polymorphism results in the so-called *cim* (class I modifier) phenomena,[160] which affects the peptide specificity of the rat TAP complex.[154, 161] The *cim*^{a} and *cim*^{b} TAPs differ by an exchange of 25 amino acids in TAP2. Two of the 25 amino acids are located in the NBD, whereas the remaining 23 are part of the TMD. Rat *cim*^{a} alleles preferentially transport peptides with a basic amino acid at the C terminus but accept also peptides with acidic, hydrophobic, aromatic, and polar side chains. In contrast, the *cim*^{b} allele is permissive only for peptides with hydrophobic, preferentially aromatic amino acids at the C terminus.[154] This polymorphism and the resulting functional difference of both alleles exert a profound effect on the assembly of class I MHC peptide complexes and the recognition by CTLs.[160, 162]

In the next step, the sequence specificity of TAP from various species was investigated. Model peptides with a glycosylation consensus sequence were investigated to establish specificity patterns for various allelic variants of rat, mouse, and human TAP.[152, 154, 161, 163] For all species, the C terminal peptide residue determined TAP selectivity.[154, 163] Mouse and rat TAP^{u} (*cim*^{b}) prefer hydrophobic and accept also aromatic C-terminal peptide residues,[154, 164] whereas human TAP and rat TAP^{a} (*cim*^{a}) accept every residue except Pro at the C terminus.[115, 154, 161] All other

residues of the peptide show no or only a negligible influence on transport efficiency.

By using microsomal binding and transport assays, 250 peptides were assayed for affinity and transport ability.[165] From these data, a putative and somewhat different peptide-binding motif of human TAP was derived. Interestingly, residues that serve as MHC class I anchors influenced peptide affinity for TAP significantly. TAP preferred hydrophobic amino acids in position P3 and hydrophobic or charged residues in position P2. From these data, human TAP specificity appears to be mainly dictated by the N-terminal amino acids. It was also shown that proline in position P3 had a negative effect on peptide transport by murine TAP.[166] Comparing the proposed putative binding motif of TAP with human MHC class I binding motifs revealed a puzzling question. Certain alleles such as HLA-A1, HLA-B7, or HLA-B8 possess primary anchor residues that have a low affinity for TAP, and therefore they show a low transport efficiency. This implies that either a putative peptide-binding motif of TAP that is based on 250 peptides only is somewhat premature or that trimming of longer peptides generates shorter peptides that will bind to the above-mentioned MHC alleles.

4.3. Deciphering the binding motif of human TAP by combinatorial chemistry

Just imagine the millions of different peptides presented by class I MHC molecules. The majority of them are transported by TAP. Consequently, a "key – lock mechanism" for peptide recognition is hard to imagine. Maybe a somewhat flexible binding site for peptides exists, which accept basically every peptide regardless of its sequence. Alternatively, a binding principle similar to MHC molecules might exist. Because solving the molecular principles of TAP affinity by conventional approaches is simply impossible, combinatorial chemistry was applied to determine the binding motif of human TAP.[133, 167] Only the binding step was investigated to minimize the above-mentioned pitfalls of the transport assays. In the library used, each position (X) contains all natural amino acids except cysteine in equal frequency (Figure 9). This results—at least theoretically—in $19^9 = 322\,687\,697\,779$ different nonapeptides.[167] Based on this tremendous amount of experimental data (Figure 10), observed differences between a high-affinity peptide and a completely randomized peptide were only 45-fold. For comparison, a factor of 200-fold was observed for a class I MHC restricted octapeptide using a similar combinatorial library.[168] Thus, human TAP seems to be less restrictive than class I MHC molecules. With this combinatorial approach, the binding motif for human TAP was derived.[167] The principle of the positional screening employing peptide libraries is explained in Figure 10 (for review see ref. [169]). The strongest selectivity was observed for the C-terminal residue. Here, hydrophobic or basic residues (Phe, Leu, Arg, or Tyr) are preferably bound. Asp, Glu, Asn, and Ser are disfavored. In addition, a preference for certain residues at the N terminus was detected. At position P1, Asn and to a smaller extent Lys and Arg, at position P2, Ile, Gln, and Arg, and at position P3, Tyr and to a smaller extent Trp are favored. Drastically lower affinities were observed for Asp and Glu

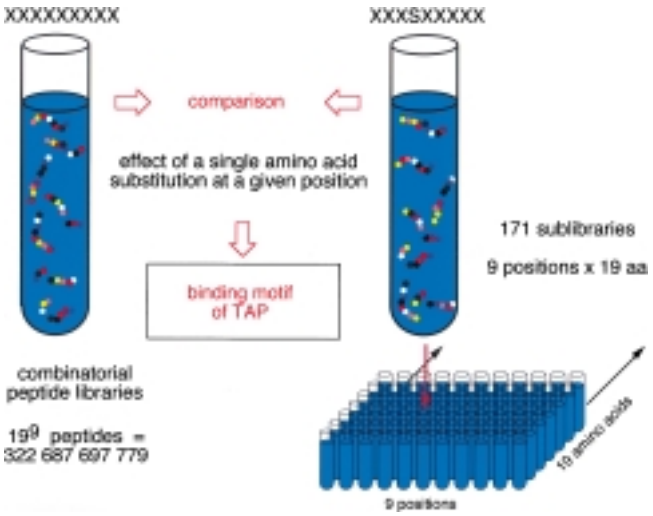

Figure 9. *Schematic principle of the combinatorial peptide libraries used to determine the peptide-binding motif of TAP. The combinatorial peptide libraries consisting of (at least theoretically) 322 687 697 779 peptides are subdivided into sublibraries. In a given sublibrary one position of the nonameric peptide is kept constant whereas the remaining eight positions randomly contain the other 19 natural amino acids in equal distribution (X). By comparing individual sublibraries ($19 \times 9X_8O$) with the complex library (X_9) the effect of each individual amino acid with respect to its position in the peptide sequence can be derived. By taking all these data together, the binding motif of human TAP is established.*[133, 167]

(position P1), Pro (position P2), and Asp, Glu, and Gly (position P3). Especially, the selection pattern of TAP for the C-terminal residue correlates well with the binding specificity of human class I MHC molecules. The selection principles at the N terminus are less clear. Only Pro, which is an anchor residue for certain HLA-B alleles, has a clearly destabilizing effect on peptide binding to TAP. On the one hand, this observation argues for an ER-resident trimming machinery. On the other hand, the negative influence of Pro pointed to a special role of the peptide backbone in peptide – TAP interactions.

To investigate this hypothesis, libraries were created that contained D-amino acids at various positions.[167] The strongest effect was observed for the first three N-terminal residues. A minor effect was detected for the C-terminal amino acid. To further study the importance of the termini, "inverted" and "retro-inverted" peptides were used (Figure 11). The inverted peptide had a sequence identical to that of a peptide with medium binding affinity ($K_D = 0.146\ \mu M$) for TAP, but contained the corresponding D-amino acids. Consequently, the orientation of the side chains of the inverted peptide is mirrored with respect to the natural peptide. No stable inverted peptide – TAP complex formation was detectable ($K_D > 1000\ \mu M$). Thus, the proper orientation of the amino acid side chain of at least some residues in the peptide is a prerequisite for binding. To extend this analysis further, a "retro-inverted" peptide was studied. In addition to the use of D-amino acids, the sequence was inverted. In such a peptide the side chains have the proper three-dimensional orientation, but the charge of the termini and the peptide backbone are inverted. As for the inverted peptide, no stable complex formation was observed for the retro-inverted

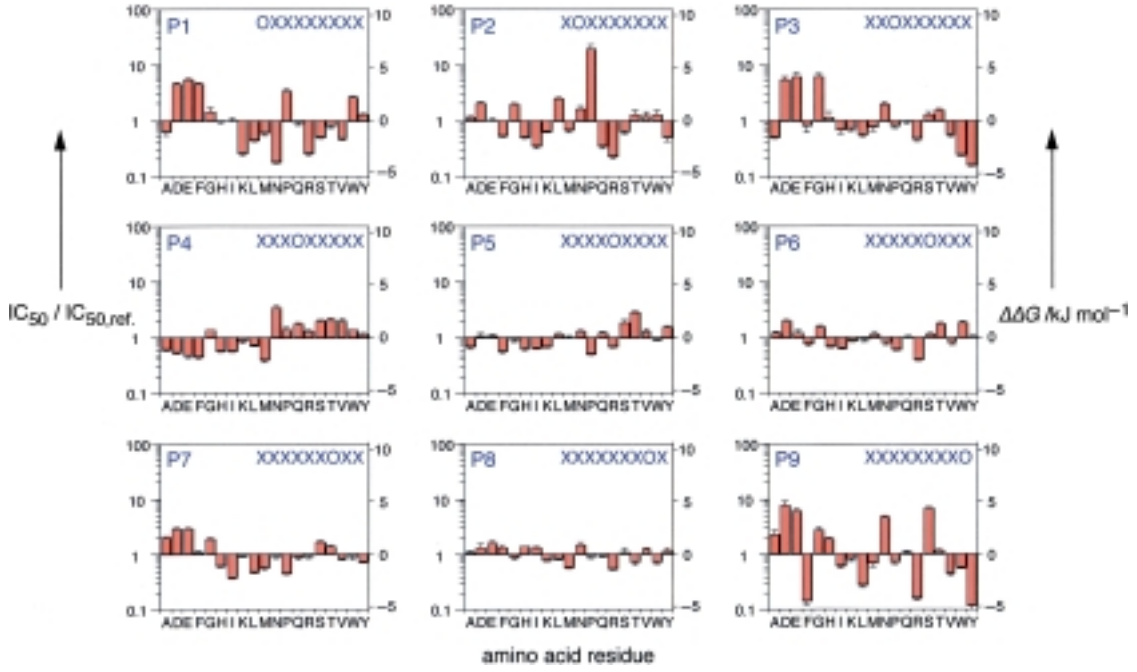

Figure 10. *Summary of studies of peptide binding to human TAP using combinatorial peptide libraries. Shown is the dependence of the amino acid at a certain position (P1 to P9) of the nonameric peptide employed in the binding assay on its ability to compete with a reporter peptide ($IC_{50}/IC_{50,ref}$) and on the stability of the resulting TAP–peptide complex (ΔΔG).*

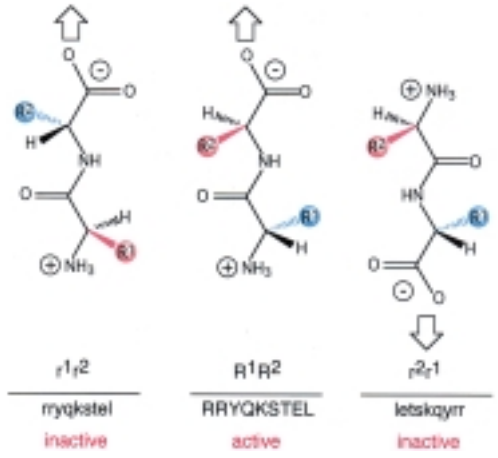

Figure 11. *Interplay between configuration and sequence of a peptide on its capability to form a stable peptide–TAP complex. Incorporation of D-amino acids creates an inverted peptide (r^1r^2, peptide on the left) that does not form a stable complex with TAP. Here, the orientation of the amino acid side chains is inverted. The retro-inverted peptide (r^2r^1, peptide on the right) is generated by mirroring the sequence and using again D-amino acids. Here, the orientation of the amino acid side chains is identical to the "wild-type peptide" (R^1R^2, peptide in the middle), but the termini and the peptide backbone are inverted. As for the inverted peptide, no stable complex formation was observed.*

peptide ($K_D > 1000$ μM). Further studies with a randomized library of dodecameric peptides and a pentadecamer with defined sequence containing D-amino acids revealed the same binding principles. Again, residues 1 to 3 as well as the C-terminal residue were of major importance for binding to TAP.[167] These results also explain on a molecular level why TAP tolerates peptides containing bulky side chains such as fluorescent labels at certain positions.[133, 170, 171] A sterically flexible substrate-binding site will allow the incorporation of various kinds of biophysical probes to determine structural aspects of the peptide-binding site.

In summary, this set of experiments clearly established that peptides are hydrogen-bonded through their free N and C termini and the peptide backbone of residues 1–3 (Figure 12). The side chains at the C terminus and positions 1, 2, 3 contribute

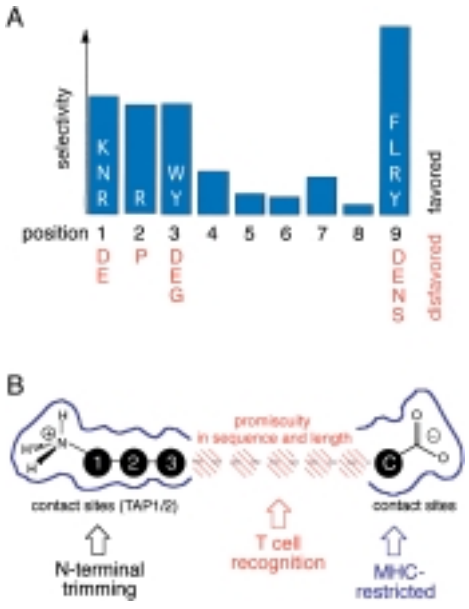

Figure 12. *Peptide-binding motif of human TAP as derived from combinatorial peptide libraries. The results of the peptide binding assays are summarized (A). A potential binding pocket including anchor residues and residues involved in TCR recognition are given (B). For further details see text.*

to the binding affinity. Consequently, the selection process of TAP occurs in regions of the peptide that are not involved in TCR recognition.[12, 13] This principle guarantees that nearly every possible TCR recognition sequence will be transported and presented with equal efficiency. The lack of either side chain specificity or backbone modification at positions 4 – 8 explains why no clear cutoff for longer peptides was determined. The absence of detectable contacts between the protein and the peptide indicate that longer peptides "bulge" out of the peptide-binding region of TAP. Thus, peptide selection by TAP and TCR recognition do not interfere with each other and place minimal restrictions on the pool of peptides available for antigen presentation (Figure 12).[172]

4.4. Mechanisms of peptide binding and transport

Most of the kinetic and thermodynamic parameters for CFTR and MDR1 were derived from reconstituted in vitro systems such as proteoliposomes (for a summary see refs. [173 – 177]). These data provided further insights into the molecular principles underlying the biological function of these proteins. Mechanistic studies of TAP are hampered by the fact that no isolated or reconstituted model systems exist. So far only two procedures for the reconstitution of TAP into liposomes have been described.[149, 150] Thus, the determination of kinetic and most of the thermodynamic properties was exclusively derived from microsomes or semipermeabilized cells.

Analysis of the kinetics of peptide translocation revealed that TAP function could be dissected into two steps. The first step, peptide binding, is ATP-independent, while the second one, peptide translocation, is ATP-dependent.[114, 133] Dissociation constants for various peptides determined directly by binding assays or indirectly by competition assays were in the submicromolar range.[114, 133, 153] All of the peptides investigated displayed a noncooperative interaction indicating a single peptide-binding site. In addition, neither the K_D value nor the saturation value for peptide binding was affected within experimental error by the addition of ATP.[133] Recently the kinetic properties of the ATP-driven peptide translocation process of murine TAP in microsomes were studied.[178] Based on a Michaelis – Menten analysis, a K_M value in the micromolar range (0.66 ± 0.09 μM) and a V_{max} value of 2.91 ± 0.25 fmol min^{-1} per μg of microsomes were determined. Although the concentration of peptides in the cytosol has not been determined, this value will definitely be below 1 μM. Thus, a K_M value around 1 μM should enable TAP to react to small changes in the concentration of cytosolic peptides. A sudden increase in peptide concentration will result in immediate transport and loading onto class I MHC molecules.

Transport assays employing (sub)cellular systems proved the strict requirement of ATP hydrolysis for peptide translocation. Accordingly, nucleotide depletion by apyrase and the use of nonhydrolyzable ATP analogues completely abrogated transport.[107, 111–113] These data clearly demonstrated that hydrolysis and not merely ATP binding is a prerequisite for translocation. In addition to ATP, GTP and UTP have been demonstrated to act as energy sources for TAP, which implies that no clear preference of nucleotides for TAP exists.[111, 179] Furthermore, one can speculate that a mechanism in which peptide binding generates a signal that triggers ATP binding and hydrolysis might act as a protective shield against a high basal ATPase activity of TAP and therefore a waste of ATP, as is known for hexokinase and other kinases.[180]

Recently it has been demonstrated that nucleotide binding has a second function in stabilizing the TAP complex.[181] These findings again point to a strong communication between NBDs and TMDs. In addition, a peptide-stimulated ATPase activity of the TAP complex reconstituted in liposomes was observed,[182] and a striking correlation between peptide affinity and ATPase activity was found. Parallel, time-resolved fluorescence spectroscopy was applied to investigate the molecular steps of peptide binding in detail.[171] A two-step binding process of fluorescence-labeled peptides to TAP was observed in the absence of ATP. The data indicate that formation of the TAP – peptide complex is composed of a fast, initial association step followed by a second, slow isomerization step of the TAP complex. This structural switch implies that TAP adopts a substrate-induced intermediate state before the ATP-driven translocation step occurs, again pointing to a strong communication between TMD and NBD (Figure 12). The importance of a crosstalk between NBD and TMD has been described by Knittler et al.[39] Mutations of the two glycines in the Walker A motif of TAP1 and TAP2 not only abolished ATP binding but also peptide-mediated release of the class I MHC molecule from the TAP complex.

4.5. Viral immune escape: TAP as target for immune suppression

The vertebrate body is an ideal breeding ground for all kinds of viruses. The conditions encountered there promote their growth, survival, and reproduction. On this battlefield, the immune system is challenged. Under the evolutionary pressure of an effective antigen processing machinery and the cellular immune system, viruses have developed fascinating strategies to win this daily battle. Some viruses simply "hit and run", completing replication before the immune response has been fully aroused. Other viruses have elaborated more sophisticated ways to avoid, evade, or even modify an immune response to their advantage (for a review see refs. [179, 183]). It is very likely that viruses target every key molecule of the cellular immune system. For example, the Epstein – Barr virus encodes its own cytokine.[184] Some viruses neutralize the action of these important immune system mediators, others eliminate class I MHC molecules from the cell surface, thereby generating cells that are temporarily invisible for cytotoxic T lymphocytes. Finally, some viral strategies are directed against TAP. By disrupting TAP-mediated translocation of antigenic peptides over the ER membrane, parts of the cellular immune response fail. Identification of these actions will help to decipher antiviral immunity. But even more importantly, understanding the mechanisms of host – virus interaction will guide vaccine development by taking advantage of millions of years of evolution.

One of the most widespread viruses is the herpes simplex virus (HSV). Probably 30 to 50 % of the human population are

infected. HSV type 1 and 2 replicate in superficial cells such as those of the lips or genitals. After the initial attack, HSV establishes a latent infection in epithelia cells of neuronal tissues. Here the virus is more or less sheltered from the cellular immune system, and it is therefore very unlikely that the virus will be eliminated. The virus abolishes the immune response by actively perturbing class I MHC mediated antigen presentation.[185] A small cytoplasmic protein, ICP47, achieves this blockage by disrupting peptide translocation across the ER membrane.[186, 187] Inhibition of TAP results in empty class I MHC molecules at the cell surface and therefore enables the virus to escape immune surveillance. ICP47 is a small polypeptide of 88 amino acids (10 kDa) with no sequence similarity to any other known protein. Interestingly, ICP47 shows high species specificity. Whereas human TAP activity was efficiently inhibited by ICP47, a 100-fold reduced affinity was observed for murine TAP.[188, 189] Studies with TAP-enriched microsomes revealed that ICP47 interacts with TAP on the cytosolic side. The determined affinity constant ($K_D =$ 50 nM) indicated a stronger interaction with TAP than for most of the peptides studied so far.[188] Thus, the molecular mechanism of ICP47 seems to involve competition for the peptide-binding site. By occupying this site, the first and essential step in peptide translocation is blocked.[188, 189]

In addition to its unique amino acid sequence, ICP47 displays fascinating structural features. First observations indicated that the viral protein could be heated to 100 °C or dissolved in chloroform/methanol without losing its activity. Membrane mimetics induce a conformational change from a predominantly random-coil state to a protein with high α-helical content.[190] Lipid-induced formation of an amphipathic α helix has been widely described as mode of action for signal sequences[191] and peptide hormones.[192] Thus, this switch implies that the interaction of ICP47 with TAP is mediated by the amphipathic helix. To further examine the molecular principles of the ICP47 – TAP interaction, truncated versions of ICP47 were generated and examined.[193] C- and N-terminal deletions revealed that the active domain is composed of residues 3 to 34. More importantly, three charged residues (residues Asp24, Lys31, and Arg32) in the active domain were identified as critical to function. The distance between two of them in the sequence (seven amino acids) mimics closely the optimal length preference of TAP. Thus, one might speculate that both amino acids resemble the free N and C termini of natural peptide substrates. Alanine scans of the N-terminal core region indicated that three stretches (residues 8 – 12, 17 – 24, and 28 – 31) are important for TAP inhibition.[194] Of these stretches, the middle one around residues 17 – 24 showed the least tolerance for alanine substitutions. Both ICP47 and the N-terminal, active domain of ICP47 (3 – 34) bind to both TAP subunits and the putative peptide-binding site as demonstrated by photo-crosslinking.[188, 189, 194] Further studies indicated that ICP47 weakens the interaction between the two TAP subunits. The induced "loose" association within the heterodimer most likely contributes to the inability of peptide translocation, simply by modifying the structure of the peptide-binding site.[195]

Recently, the NMR structure of the active domain of ICP47, ICP47(2 – 34), has been determined in detergent solution.[196] In contrast to the situation in aqueous solution, in which ICP47 possesses no detectable secondary structure, ICP47(2 – 34) adopts a helix-turn-helix motif in SDS micelles (Figure 13). The authors propose that the observed helix-turn-helix motif is a requirement for the binding of one α helix to TAP while the other

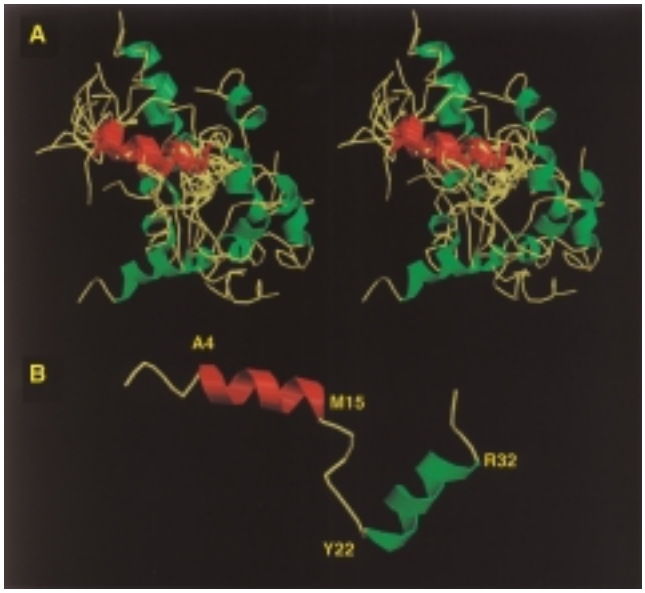

Figure 13. *A: Stereoview of ten NMR structures of ICP47(2 – 34) in the presence of the membrane mimetic SDS.[196] B: One of the structures from Figure A, with both helices drawn in red and green and unstructured parts in orange. Amino acids starting and terminating the helices are indicated using single-letter codes; their numbers are also given to indicate the position in the sequence.*

one remains bound to the membrane. This view is also supported by an earlier model, in which ICP47 does not penetrate the membrane. Rather it is located at the membrane surface or the protein – lipid interface, thereby inhibiting TAP (Figure 13).[190]

Another virus that establishes a lifelong infection is the human cytomegalovirus (HCMV). In contrast to HSV, it infects a wide range of cells and consequently has to use a very complex strategy to prevent class I MHC presentation of viral proteins.[197, 198] Based on the diversity of the host cells of HCMV, this virus has developed various strategies to escape immune surveillance. For example, a general arrest of class-I-mediated immune surveillance is achieved by several gene products of the unique short (US) region.[199] The recently identified glycoprotein US6 inhibits peptide loading of class I MHC by interacting with TAP.[197, 200, 201] US6 is a type I transmembrane protein with a molecular mass of 21 kDa and a single N glycosylation site. In contrast to ICP47, US6 blocks TAP from the luminal side of the ER.[197, 201] Thus, inhibition is achieved without any participation of the putative peptide-binding site of TAP. In contrast to peptide translocation, peptide binding to TAP is not inhibited by US6. These results point to an unknown control mechanism of TAP, which is used by the virus to undermine the cellular immune system.

Recently, a third viral strategy to evade the immune system by targeting TAP has been described. In addition to its function in

the ER retention of MHC class I molecules,[202, 203] the adenovirus E3 19-kDa protein (E3/19K) blocks tapasin binding to TAP.[204] The type I glycoprotein E3/19K binds separately to MHC and TAP and therefore causes a decrease in MHC – TAP association by mimicking tapasin and delaying class I MHC maturation.

Understanding the molecular events of ICP47, US6, and E19 interaction with TAP (Figure 14) is of major interest to decode the principles by which viruses escape immune surveillance. But these principles might be also used to treat immune diseases, xenograft rejection, or even induction of immune tolerance. For example, ICP47 is a very potent and specific immune suppressor.

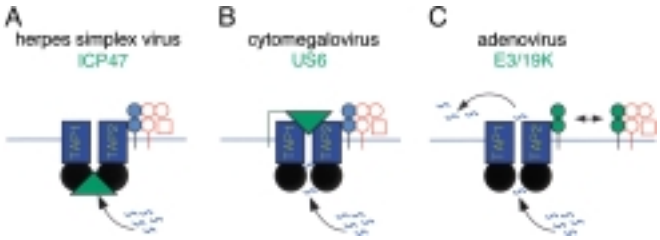

Figure 14. *Immune evasion strategies of viruses using TAP as a target. A: The HSV protein ICP47 binds to the cytosolic face of the TAP complex, thereby preventing peptide binding to TAP. B: The type I glycoprotein US6 of HCMV interacts specifically with the ER-luminal face of TAP and abolishes peptide transport by a so far unknown mechanism. C: The type I glycoprotein E3/19K encoded by adenovirus type 2 substitutes tapasin in the "loading complex" and prevents physical contact between TAP and class I MHC molecules. Peptides are still transported into the lumen of the ER, but not loaded efficiently onto class I MHC molecules.*

Designing a synthetic ICP47 analogue would combine affinity and specificity. The affinity would ensure that TAP is targeted specifically, and functional shutdown would be achieved in a controlled fashion. The specificity would exclude any side effects commonly encountered with synthetic drugs. Thus, an immune suppressor without harmful side effects would be available for the first time. A similar scenario could be imagined for US6. Again a synthetic mimetic would allow to perturb class I MHC mediated antigen presentation, but without side effects. However, to achieve such a task, an understanding of the host – virus interactions and the underlying mechanistic aspects is essential.

5. Conclusions and outlook

Over the past decade, a tremendous amount of data has been accumulated that enhanced our understanding of the cellular immune system at the molecular level. In case of class I MHC mediated immune surveillance, it was shown that TAP, a member of the exponentially growing family of ABC transporters, plays an essential role. This membrane protein translocates antigenic peptides across the ER membrane into the ER, where they are loaded onto nascent class I MHC molecules. TAP function was demonstrated in a set of elegant experiments. Subsequently, it was shown that peptide transport is composed of a series of independent steps. The first step, peptide binding to TAP, is ATP-independent, while the second one requires ATP or other nucleotide triphosphates for energizing of translocation. Sequence comparisons with other members of the ABC transporter family revealed a high homology within the NBDs, but a large divergence in the TMDs. This observation supported the idea that the substrate-binding site is located near or within the membrane-spanning part. Photolabelling studies proved that both subunits of the TAP complex form the peptide-binding site. By applying combinatorial chemistry, it was possible to extract a peptide-binding motif from a library of billions of nonapeptides. The putative motif resembles the principles of MHC – peptide recognition in which only a subset of potential contact sites is actually used to generate stable binding. In case of TAP, the termini as well as the first three N-terminal and the C-terminal side chains are employed to pin down the peptide with high affinity. The residues in between are the ones that are recognized by the TCR. This principle ensures maximal diversity for the pool of antigenic peptides presented at the cell surface.

All of these results, which are the basis for our current understanding of the molecular function of TAP, have been derived from studies with microsomes or semipermeabilized cells. Here, a somewhat ill-defined situation is encountered. Neither purity nor amount of TAP nor presence of export systems, proteases, or an ER-resident trimming machinery can be controlled. Thus, those results have to be interpreted with great caution. But in the absence of an artificial model system in which the above-mentioned parameters can be controlled, these seminatural systems provide important insights. Nevertheless, proteoliposomes containing isolated TAP will open up new possibilities to study mechanistic and structural aspects of this ABC transporter. With such a model in hand, kinetic and thermodynamic studies can be performed under various conditions. The results obtained will complete our understanding of the principles of TAP function. Open questions, such as the communication between the NBD and TMD, the conformational changes upon peptide or ATP binding, the interactions between TAP, several cofactors of the macromolecular peptide transport and loading complex, and viral effectors, will be answered. Interesting times and a lot of astonishing results will probably lie ahead of us.

Our apologies go to all colleagues whose work is not referenced appropriately. We are indebted to all current and former members of our group and to our collaborators. Without their enthusiasm and work, many insights into TAP function and mechanism would not exist. We also thank Dr. S.-H. Kim, UC Berkeley, for providing the coordinates of HisP prior to release from the Brookhaven Protein Data Bank. We especially thank Dr. M. T. Stubbs for his constant support and assistance during the protein modeling studies. The Deutsche Forschungsgemeinschaft (DFG) and the Fonds der Chemischen Industrie (FCI) funded research in our laboratory.

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