Molecular and Functional Characterization of Early and Late Checkpoints in the Quality Control of MHC Class I-Restricted Antigen Presentation

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1. Introduction

1.1 The organization of the mammalian immune system

The mammalian immune response can generally be subdivided into three interdependent limbs, namely the innate immune response, the humoral response and the cell-mediated response. The innate immune system is believed to provide a first barrier against the spread of pathogens within a newly infected host. Thereby it counteracts the establishing infection and contributes to an early clearance of the pathogen. However, the term “innate immune response” describes a very diverse repertoire of mechanisms, among them complement-mediated killing of bacteria (Gasque et al, 2004), secretion of peptide antibiotics (Boman et al, 1995), elimination of extracellular pathogens through phagocytic uptake by macrophages (Hingley-Wilson et al, 2000) and NK-cell mediated cytotoxicity (Ljunggren et al, 1990). The innate immune system identifies its microbial targets mainly by the recognition of relatively invariable so-called pathogen-associated molecular patterns (PAMPs) (Janeway et al, 2002) through a huge number of specialized receptors, including the Toll-like and NOD family of receptors (Janeway et al, 2002; Tschopp et al, 2003). Numerous cell types including macrophages, NK cells and epithelial cells contribute effector functions to this limb of the immune system and in addition many (perhaps all) non-immune cells can acquire an antiviral state upon γ-interferon treatment, a phenomenon commonly referred to as cell-autonomous resistance (MacMicking et al, 2004; Weber et al, 2004). The humoral immune response is mediated by the B cell population, which secretes an enormously diverse repertoire of antibodies (Calame et al, 2003). Antibodies neutralize toxins (Little et al, 1988; Wild et al, 2003), block the adhesion of pathogens to mucosal surfaces (Kunisawa et al, 2005) or opsonize pathogens to mark them for phagocytic uptake (or immediate destruction) (Stuart et

al, 2005). Thus, an antibody-mediated immune response is predominantly directed against extracellular pathogens. According to the theory of clonal selection each individual B cell produces only a single type of antibody. The mature gene that encodes an antibody is generated by rearrangement of DNA (Sadofsky et al, 2001) and its expression gives either rise to the B cell receptor (BCR), which is anchored in the plasma membrane or to the secreted antibody, which is its soluble analog (Cambier et al, 1992). Antibodies can recognize the antigens they are targeted against in their native conformation. This distinguishes them fundamentally from the related T cell receptors (TCRs) which require both processing of their epitopes and presentation of their peptide antigens in the context of a major histocompatibility (MHC) class I or II molecule (Myers et al, 1991). TCR diversity is generated by a site-directed DNA recombination process largely similar to the mechanism that generates the high variety within the antibody repertoire (Sadofsky et al, 2001). T cells originate from precursor stem cells in the bone marrow and migrate during their early development to the thymus where they differentiate (Bommhardt et al, 2004). There they undergo both positive as well as negative selection with the consequence that only MHC-restricted cells survive that do not recognize self-antigen with high affinity (Sebzda et al, 1999). MHC class II-restricted T cells become selected to the $CD4^+$ lineage and henceforth recognize peptide antigens that (largely) derive from extracellular proteins ingested by professional phagocytic cells. These include dendritic cells, macrophages and B cells. Accordingly, these cell types express high levels of MHC class II. The proteolytic processing of MHC class II-presented antigens occurs in compartments along the endocytic route (e.g. endosomes or lysosomes) and requires the activity of a diverse set of enzymes, among them cysteine proteases (e.g. cathepsins S, L, B, H and F) and the γ-interferon-induced lysosomal thiol reductase (GILT) (Watts et al, 2004). Two major $CD4^+$ lineages exist: inflammatory

T_H1 cells and T_H2 helper cells. The former secrete γ-interferon and activate macrophages to become bactericidal whereas the latter activate B cells to secrete antibodies (Dong et al, 2000). Thus, $CD4^+$ T-lymphocytes represent an important connection between cell-mediated immunity on the one hand and innate or humoral immune response on the other hand. In contrast, MHC class I-restricted T cells become selected to the $CD8^+$ lineage. The vast majority of antigenic peptides that are recognized by CD8-positive T cells in combination with MHC class I are breakdown products of the proteolytic degradation machinery in the cytosol (Leonhardt et al, 2003; Pamer et al, 1998). Accordingly, the cell-mediated immune response exerted by cytotoxic ($CD8^+$) T lymphocytes (CTLs) is mainly directed against intracellular pathogens, primarily viruses and some bacteria. In addition CTLs make a major contribution to the elimination of tumor cells. In line with this, MHC class I molecules (in contrast to their class II counterparts) are expressed on the surface of all nucleated cells. The recognition of non-self peptide by a CTL at the surface of an infected (or malignant) cell leads to the release by the T cell of granules containing perforin and granzymes that have membrane-disrupting and apoptosis-inducing activity (Trapani et al, 2002). Using this pathway and/or a FasL-induced mechanism the CTL activates the apoptotic program of the infected (or malignant) target cell (Trapani et al, 2002). Therefore it is not surprising that many viral (Hewitt et al, 2003; Lybarger et al, 2005) but also bacterial (Neumeister et al, 2005; Qimron et al, 2004) pathogens have developed a huge number of immune evasion strategies that subvert the class I antigen presentation pathway. In addition, tumor cells also frequently down-modulate class I surface levels to avoid their recognition by CTLs (Bubenik et al, 2003). However, during an infection (or the onset of cancer) the three limbs of the immune system synergistically attack intruding microbes (or malignant cells) and in most cases efficiently protect the host organism from disease.

1.2 The processing of MHC class I-presented antigenic peptide ligands

As mentioned above antigenic peptide ligands for MHC class I mostly derive from the degradation of intracellular proteins in the cytosol (Pamer et al, 1998). The major endoprotease that contributes oligopeptides to this pathway is the proteasome (Fig. 1) (Goldberg et al, 2002; Kloetzel et al, 2001). Moreover, in the context of some MHC class I alleles the tripeptidyl peptidase II (TPP II) is supposed to play an

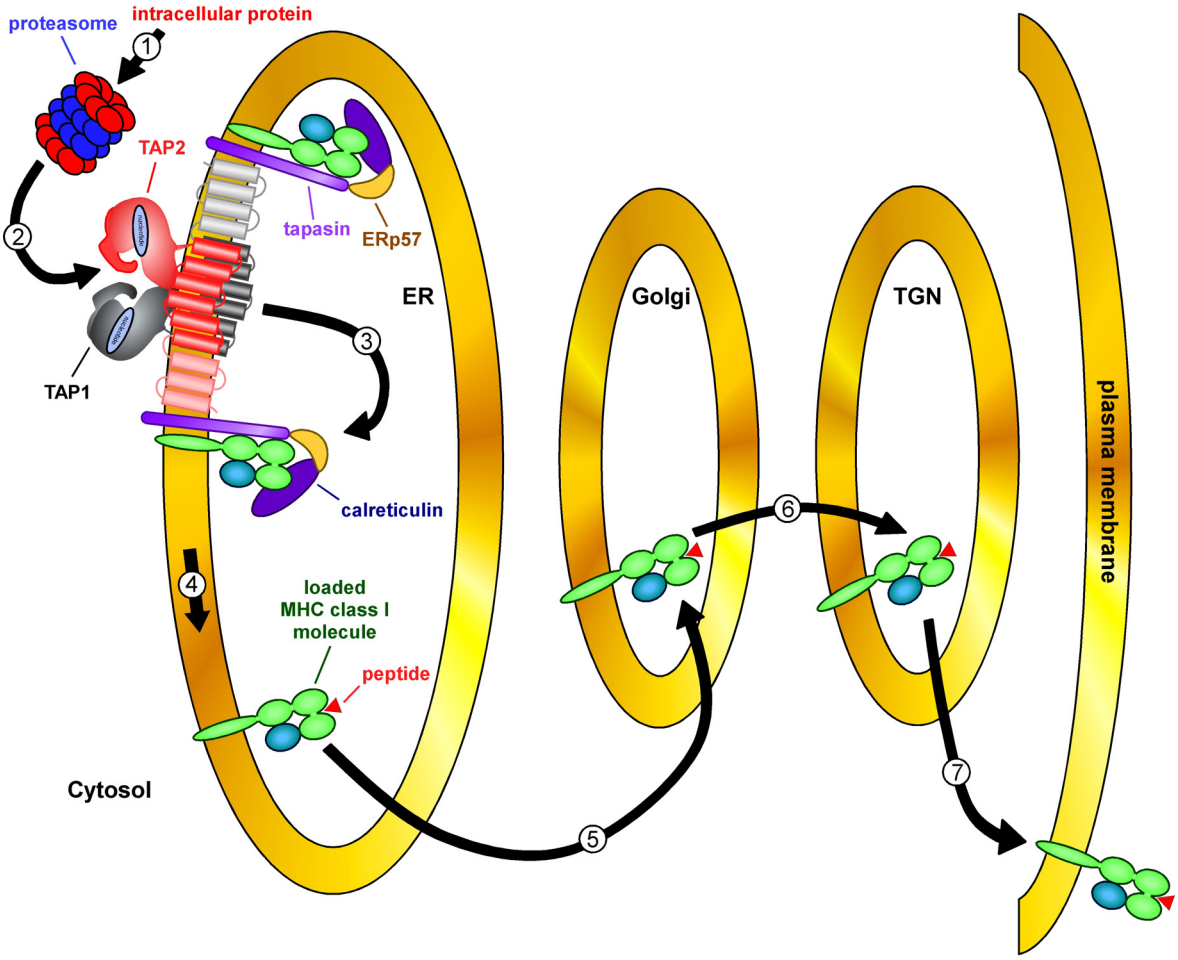

Fig. 1 **The MHC class I antigen presentation pathway**
Intracellular proteins (1) are degraded by the proteasome in the cytosol (2) and the resulting oligopeptides are subsequently translocated into the ER by the peptide transporter TAP (3). There they are loaded onto empty MHC class I molecules (3). These are at that stage part of a multi-protein complex, the so-called MHC class I peptide loading complex (PLC). Within this complex TAP is bridged to MHC class I by tapasin. Additionally the PLC comprises the lectin chaperone calreticulin and the oxidoreductase ERp57. Upon binding of a peptide ligand the loaded MHC class I molecule dissociates from the PLC (4) and migrates to the plasma membrane (7). On this way it passes the Golgi apparatus (5) and the *trans*-Golgi network (6).

important role for the endoproteolytic generation of antigens (Seifert et al, 2003). However, proteasomal activity yields only a small fraction of peptides that have the

appropriate length of 8 to 10 amino acids to bind an MHC class I molecule. Namely, more than 70% of the oligopeptides generated by the proteasome are too short, whereas 15% exceed the size of a suitable class I ligand (Kisselev et al, 1999). Aminopeptidases can trim the latter population to a fitting length (Goldberg et al, 2002). Currently, for several cytosolic aminopeptidases a role in the MHC class I antigen presentation pathway is discussed, among them puromycin-sensitive aminopeptidase (Levy et al, 2002; Stoltze et al, 2000), bleomycin hydrolase (Stoltze et al, 2000), TPP II (Levy et al, 2002) and γ-interferon inducible leucine aminopeptidase (LAP) (Beninga et al, 1998). Furthermore, upon induction with γ-interferon the proteolytic machinery in the cytosol becomes modified (presumably) for adjusting it to the requirements of the immune system in the context of an infection. Particularly, the composition of the proteasome changes by the incorporation of three new γ-interferon-induced subunits and the regulator PA28. The resulting so-called immunoproteasome is distinct from the regular housekeeping proteasome with respect to its cleavage specificity and hence the peptide repertoire that it generates (Cascio et al, 2001). Consistently, the production of several important viral or tumor-associated peptide antigens specifically depends on the activity of this modified protease (Cerundolo et al, 1995; Lautscham et al, 2003; Schultz et al, 2002) or the presence of PA28 (Sijts et al, 2002). Interestingly, the activity of the immunoproteasome was shown to yield more N-terminally extended precursors of an intensively investigated model peptide ligand for MHC class I when compared to its constitutive counterpart (Cascio et al, 2001). However, the finding that the incorporation of the immuno-subunits into the proteasome conversely abrogates the production of some epitopes (Morel et al, 2000) queries the view that immunoproteasomes are always the “better” proteasomes under all circumstances. During the early phase (approx. one week) of a viral or bacterial infection constitutive

proteasomes were reported to become gradually replaced by their immuno-counterparts (Khan et al, 2001). Thus, the combined presence of both in the beginning of the pathogenic challenge may broaden the spectrum of antigenic peptide ligands produced, thereby increasing the efficiency of an immune response. After their generation in the cytosol oligopeptides have to traverse the endoplasmic reticulum (ER) membrane before they can be loaded onto class I (Fig. 1). The major route used for entry into the ER is via the peptide transporter associated with antigen processing (TAP) which resides in the ER membrane (Leonhardt et al, 2003; Schmitt et al, 2000). Although some very hydrophobic class I ligands were reported to get access to class I without the need for this transporter (Lautscham et al, 2001; Lautscham et al, 2003), the vast majority of cytosolic peptides essentially depends on the activity of TAP for their translocation into the ER. This becomes evident by markedly decreased MHC class I surface levels on cells of TAP-deficient individuals as a consequence of an almost stagnant peptide supply into the endoplasmic reticulum (Gadola et al, 2000). However, some MHC class I alleles as HLA-A2 can efficiently associate with peptides derived from signal sequences in a TAP-independent manner (Wölfel et al, 2000). After transport into the ER N-terminally extended class I ligands can be further trimmed by the γ-interferon-inducible aminopeptidase ERAP1 until a suitable length for loading onto class I is reached (Saric et al, 2002; Serwold et al, 2002). In the ER peptide-receptive MHC class I molecules associate with TAP and several chaperones to form the so-called MHC class I peptide loading complex (PLC) (Fig. 1 and 4) (Leonhardt et al, 2003; Wright et al, 2004). This complex retains class I in the ER as long as the loading with a high affinity peptide ligand has occurred (see below). Upon binding of such a ligand the MHC class I molecule dissociates from the PLC and migrates along the standard secretory route to the cell surface, where it presents its peptide to CTLs (Fig. 1)

(Yewdell et al, 2003). An unexpected surprise for the field was the finding that some antigenic peptides for class I can also be generated in the *trans*-Golgi network (TGN) by furin, a member of the proprotein convertase family (Gil-Torregrosa et al, 1998; Gil-Torregrosa et al, 2000). However, to what extent this pathway contributes to the overall repertoire of surface-presented peptides and how these specific ligands are loaded onto class I has remained elusive.

Taken together, MHC class I molecules present peptides derived from the degradation of intracellular proteins on the cell surface for perusal by $CD8^+$ T cells. This process allows CTLs to monitor the current protein content of a host cell and to identify (and kill) cells that harbor abnormal or non-self (e.g. of viral origin) proteins. To sacrifice a virally infected or malignant cell is a necessary evil that protects the host from spreading of the infection to other cells or the development of a life-threatening tumor.

1.3 The transporter associated with antigen processing (TAP)

The peptide transporter TAP (Fig. 2) is a member of the ATP binding cassette (ABC) transporter family (Schmitt et al, 2000). ABC transporters have been isolated from all three kingdoms of life, where they translocate diverse substances including vitamins, drugs, ions, amino acids, peptides, sugars or lipids across biological membranes (Garmory et al, 2004; Van der Does et al, 2004). Irrespective of the substrate specificity, which is clearly distinct among individual members of this protein family all ABC transporters share an identical four domain organization comprising two nucleotide binding domains (NBDs) and two transmembrane domains (TMDs) (Stefkova et al, 2004). TAP also possesses this characteristic four domain composition (Fig. 2A and Fig. 2B) (Schmitt et al, 2000). Its NBDs bind and hydrolyze ATP (Chen et al, 2003; Knittler et al, 1999; Saveanu et al, 2001; Schmitt et al, 2000)

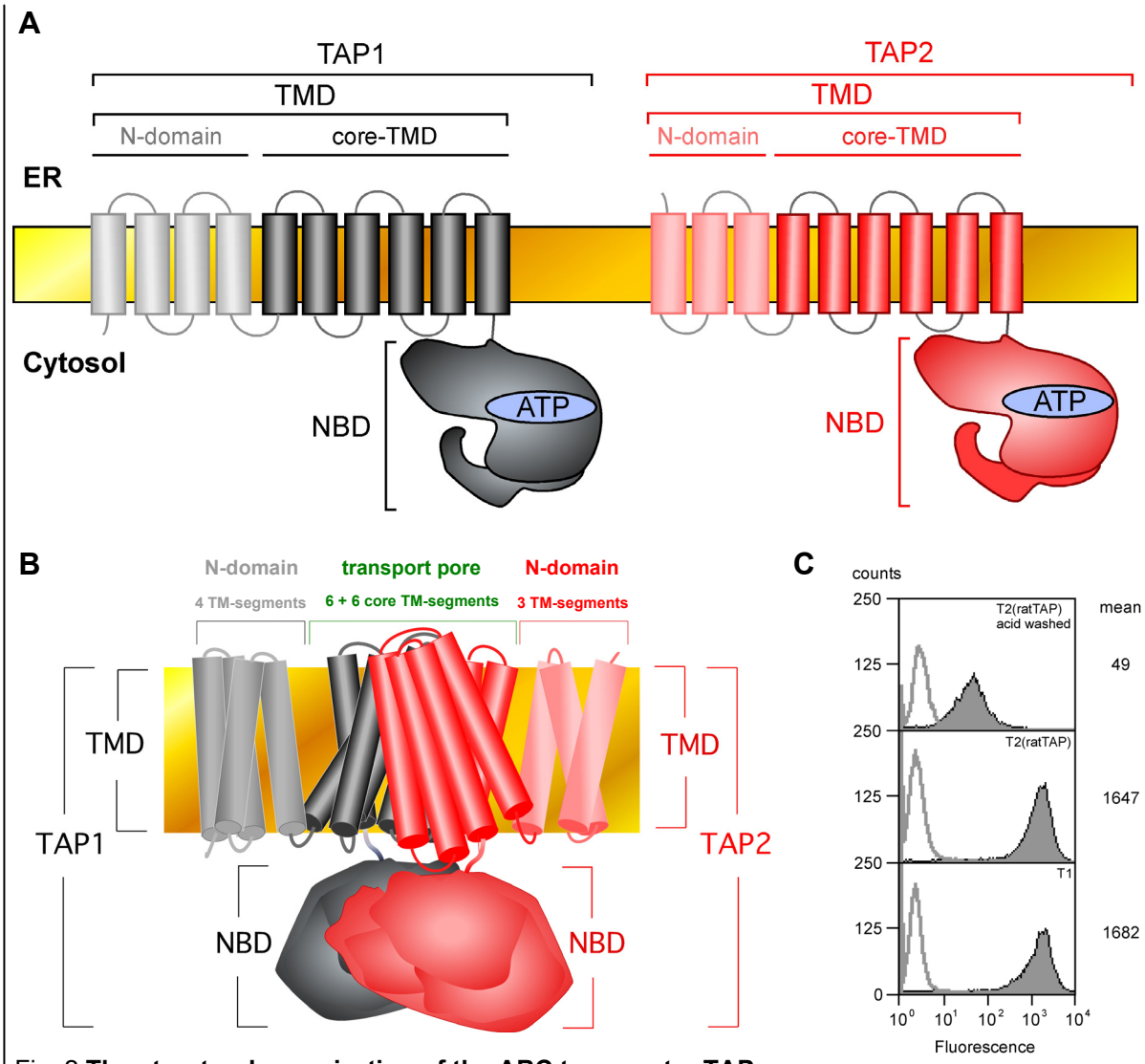

Fig. 2 **The structural organization of the ABC transporter TAP**
(A) The TAP transporter is a heterodimer that consists of the two subunits TAP1 (depicted in black) and TAP2 (depicted in red). Either chain contributes an N-terminal transmembrane domain (TMD) and a C-terminal nucleotide binding domain (NBD) to the assembled transporter. In both, TAP1 and TAP2, the TMD can be further subdivided into the N-domain at the extreme N-terminus (involved in the tapasin interaction) and an inner core TMD, comprising 6 α-helices (involved in peptide binding and the formation of the translocation pore). The NBD binds and hydrolyzes ATP to energize the peptide translocation into the ER. **(B)** Model of the TAP transport pore. The closed conformation of the lipid A-transporter MsbA from *Vibrio cholera* (Chang et al, 2003) served as a template to predict the arrangement of the putative six transmembrane segments of the TAP core TMD. **C)** MHC class I surface expression on T1 cells expressing human TAP and their TAP-deficient derivative T2, reconstituted with rat TAP^a. T1 cells or T2 transfectants were incubated with the monoclonal anti-MHC class I-antibody 4E followed by FITC-labeled secondary antibody to determine the surface expression of HLA-B5 (filled histograms). To control for the specificity of the primary antibody T2(ratTAP) cells were briefly exposed to acidic buffer for removal of assembled surface MHC class I molecules before processing the cells for flow cytometry. Background staining was analyzed by incubating with secondary antibody alone (non-filled histograms).

to energize the peptide translocation process, whereas its TMDs contain the substrate binding site and form the translocation pore (Fig. 2B; Nijenhuis et al, 1996). TAP is a heterodimer consisting of two homologous polypeptides, TAP1 and TAP2.

Each of these subunits contributes one NBD and one TMD to the assembled transporter (Fig. 2A). Both TAP chains are essential for peptide binding and transport (Arora et al, 2001; Meyer et al, 1994; Momburg et al, 1992). Sequential hydrolysis of ATP by TAP1 and TAP2 has been proposed to drive the translocation of peptide across the ER membrane and to restore the ground state of the transporter, which is characterized by high affinity for cytosolic substrates (Alberts et al, 2001). The TMDs of TAP1 and TAP2 are predicted to comprise 10 and 9 transmembrane helices, respectively (Schmitt et al, 2000). The 6 inner membrane spanning helices of both TAP subunits are designated as the core TMD, since they are sufficient to allow for the formation of a functional substrate binding site and translocation pore in the assembled transporter (Koch et al, 2004). The N-terminal extensions (N-domains) of the TMDs, (presumably) comprising 4 transmembrane helices in TAP1 and 3 transmembrane helices in TAP2 function as docking sites for tapasin (Koch et al, 2004). The preferred substrates for the transporter are peptides with a length of 8 to 12 amino acids (Abele et al, 2004). Thus, TAP most efficiently delivers those peptides into the ER that have an optimal size for binding to MHC class I. With respect to the amino acid sequence of its substrates TAP has been reported to be highly promiscuous. However, the nature of the C-terminal residue and to a minor extent of the first three N-terminal residues plays a role for peptide binding (and most likely transport) by TAP (Schmitt et al, 2000). Importantly, human TAP and rat TAP^a were both shown to be permissive for peptides with any C-terminus with the exception of proline (Schmitt et al, 2000), suggesting that both transporters translocate a similar set of peptides (Momburg et al, 1994; Pamer et al, 1998). In line with this, the human T1 cell line, which expresses the human TAP transporter and its derivative T2(ratTAP), which expresses the rat TAP^a transporter have equal levels of MHC class I at the cell surface (Fig. 2C), indicating that human TAP and rat TAP^a are

functionally equivalent. The major function of TAP is to supply ER-resident MHC class I molecules with antigenic ligands mostly generated by the proteasome in the cytosol. Since its inhibition (or absence) results in a dramatic loss of MHC class I surface levels (Ahn et al, 1997; Radosevich et al, 2003) TAP must be considered a bottleneck of the class I antigen presentation pathway in most cells. Therefore, the peptide transporter may be expected to be a major target for viral evasion strategies and indeed several viral effectors have been identified that interfere in one or the other way with the function of TAP (Lybarger et al, 2005; Momburg et al, 2002b). Taken together, TAP is a central component of the cell-mediated immune response that provides an (almost) essential connection between the proteolytic degradation machinery in the cytosol and peptide-receptive MHC class I molecules in the ER.

1.4 MHC class I molecules

An MHC class I molecule is defined as a non-covalently associated trimer consisting of the membrane-anchored MHC class I heavy chain, the soluble light chain called β_2-microglobulin and a peptide ligand, usually of 8 to 10 amino acids (Pamer et al, 1998). The heavy chain is a 43 kDa transmembrane glycoprotein and consists of three domains: α_1, α_2 and α_3. The membrane-proximal α_3-domain, as well as the β_2-microglobulin adopts a standard immunoglobulin-like fold and both comprise the conserved disulfide bond classically found in these domains (Dick et al, 2004). The peptide binding groove is part of the α_1- and the α_2-domain. The floor of this pocket consists of a β-sheet whereas either side is lined by an α-helix (Dick et al, 2004). The α_2-domain comprises the third disulfide-bridge of an MHC class I molecule (Dick et al, 2004). This disulfide-bridge lies in close proximity to the N-terminus of the bound peptide ligand (see below). The MHC class I heavy chain is both polygenic and extremely polymorphic (Adams et al, 2000). The human MHC locus on chromosome

6 encodes three different heavy chain genes, namely HLA-A, HLA-B and HLA-C (Adams et al, 2000). The human genome is diploid and hence the extensive polymorphism among the heavy chain genes promotes the expression of 6 different MHC class I alleles per individual. The association of peptide ligands with an MHC class I molecule occurs mainly through the insertion of so-called anchor residues into specific pockets in the peptide binding groove (Achour et al, 1998; Kjer-Nielsen et al, 2002). The presence of the correct anchor residues within a ligand (e.g. frequently the C-terminus) is a prerequisite for a peptide antigen to bind a given MHC class I allele. Since the polymorphism in the MHC class I heavy chain largely concentrates on the peptide binding groove, each class I allele has its particular requirements for specific anchor residues. The consequence is that different MHC class I alleles bind different sets of peptides. In this regard the expression of 6 dissimilar alleles by most individuals (and the expression of numberless alleles within a human population) increases the number of pathogenic epitopes to that the cell-mediated immune system can respond. Thus, both MHC class I polymorphism and the existence of multiple class I loci in the genome create an advantage for the organism (or in a broader sense for the population) that improves the host defense against microbial intruders as viruses or intracellular bacteria. Indeed, the presence of some class I alleles was reported to predispose individuals to (or protect individuals from) specific diseases (Jeffery et al, 2000; Schrier et al, 1995) whereas others appear to affect the course of the disease (Hendel et al, 1999). However, the class I polymorphism does not only expand the antigenic peptide repertoire presented at the cell surface for immuno-surveillance by CTLs. Additionally, MHC class I alleles were reported to substantially differ in their requirements for the presence of a functional PLC to acquire stable binding peptides (Peh et al, 1998). In this regard HLA-B5 is an example for an allele that essentially depends on the presence of tapasin for

establishing high class I surface levels (Gao et al, 2004; Grandea et al, 1997), whereas HLA-B27 appears to be virtually tapasin-independent in this regard (Peh et al, 1998). Since some viruses are known to interfere with the proper formation of a PLC (Lybarger et al, 2005), the class I polymorphism may also be part of a cellular counter strategy in providing alleles that stay functional even under these specific circumstances when viruses attempt to subvert the standard antigen presentation pathway.

1.5 Lectin chaperones, ERp57 and the oxidative folding pathway in the ER

The two lectin chaperones calnexin and calreticulin act together with the oxidoreductase ERp57 in an oxidative folding pathway for newly synthesized glycoproteins in the ER (Fig. 3) (Kleizen et al, 2004). After the initial transfer of a sugar tree to an acceptor asparagine within a nascent substrate protein the first step is that α-glucosidases I and II sequentially trim two glucose residues from the tree to generate a monoglucosylated glycan. This immature carbohydrate structure, which is typically found on newly synthesized and therefore not yet completely folded glycoproteins is subsequently recognized by either calnexin or calreticulin (High et al, 2000; Kleizen et al, 2004). Both of these chaperones associate and act in synergy together with the thiol-disulfide oxidoreductase ERp57, which facilitates the coupling of substrate oxidation and folding (Molinari et al, 1999; Oliver et al, 1999). After eventual ERp57-mediated introduction of disulfide bonds the folding cycle is completed with the action of α-glucosidase II on the substrate protein, which removes the terminal glucose on the glycan. Subsequently, the folded glycoprotein is released from calnexin or calreticulin. In the case that folding is not yet completed the glycoprotein substrate is recognized by UGGT (UDP glucose:glycoprotein glucosyltransferase), which acts as a folding sensor in the pathway and can

reglucosylate the substrate to make it reenter a new calnexin/calreticulin folding cycle (High et al, 2000; Kleizen et al, 2004).

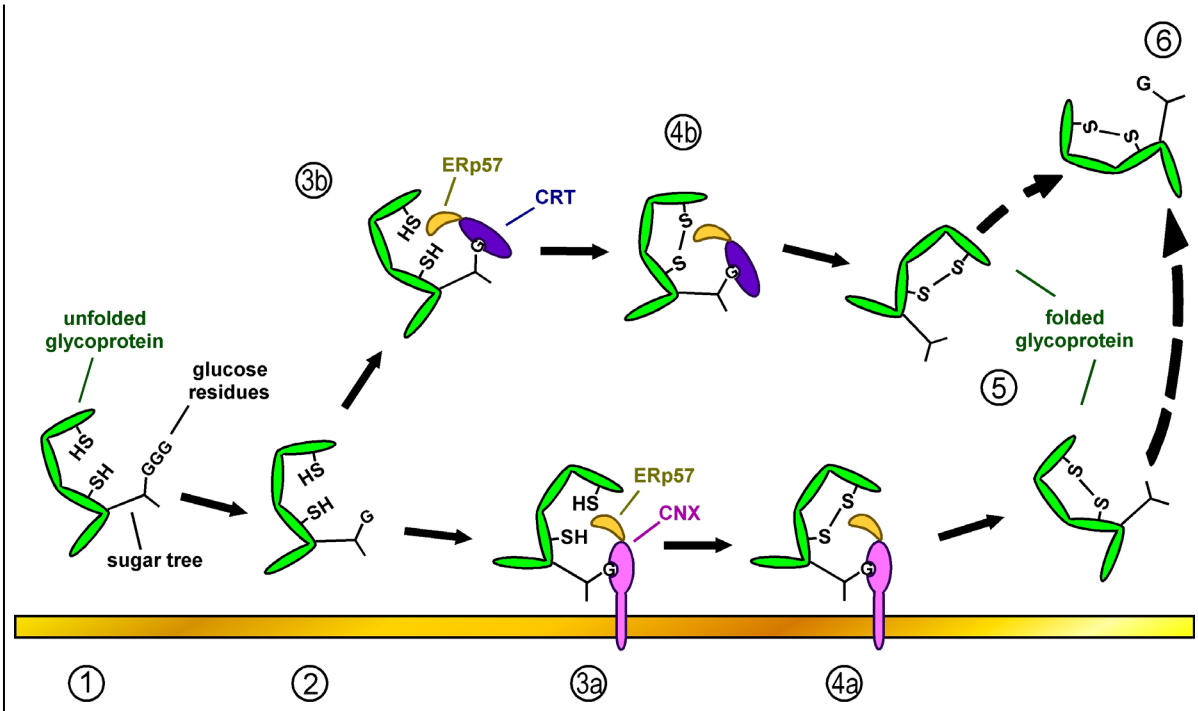

Fig. 3 **The oxidative folding cycle for glycoproteins in the ER**
Newly synthesized glycoproteins initially carry glycan precursors with the carbohydrate structure $GlcNAc_2Man_9Glc_3$ (1). This sugar tree is trimmed by the sequential action of α-glucosidases I and II, thereby generating a mono-glucosylated oligosaccharide (2). This oligosaccharide is recognized by the membrane-anchored lectin chaperone calnexin (CNX) (3a) or its soluble homolog calreticulin (CRT) (3b). Both chaperones share the capability to recruit the oxidoreductase ERp57 into the resulting folding complexes (3a/3b) that promotes the proper formation of disulfide bridges within its substrate proteins (4a/4b). The removal of the terminal glucose residue from the sugar tree by α-glucosidase II releases the polypeptide from the respective lectin chaperone (5). Reglucosylation can occur (dashed line) if the folding sensor UGGT determines that the glycoprotein is not yet properly folded (6). Subsequently, the latter can enter a new CNX/CRT-mediated folding cycle.

1.6 The assembly and function of the MHC class I peptide-loading complex

During the sequential assembly of the PLC MHC class I molecules interact at multiple stages with lectin chaperones and the oxidoreductase ERp57 (Fig. 4). The first contact occurs between calnexin and the nascent class I heavy chain, presumably to facilitate proper folding of the latter during co-translational protein translocation into the ER (Dick et al, 2004; Tector et al, 1995; Vassilakos et al, 1996). The fact that ERp57 is found in these early assembly complexes (Farmery et al,

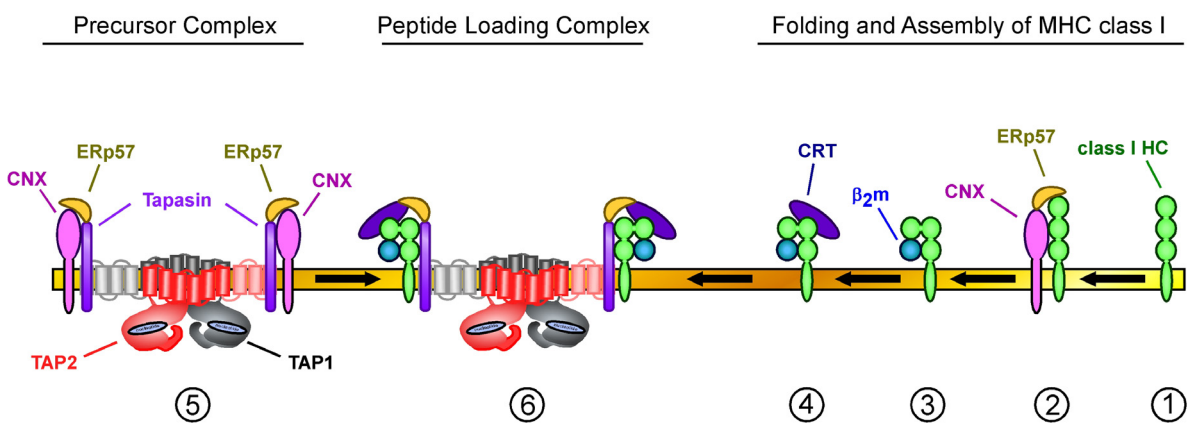

Fig. 4 **Hypothetical model on the assembly of the MHC class I-peptide loading complex** Glycosylated MHC class I heavy chains (HC) (1) associate with calnexin and ERp57 in the ER (2). Both chaperones together promote the proper oxidative folding of the HC. The subsequent interaction with β_2-microglobulin (3) leads to the disassembly of this initial folding complex through the release of calnexin (and probably also ERp57). Concomitantly with (or rapidly upon) the association of β_2-microglobulin with the HC (3), calreticulin (CRT) is incorporated (4). The resulting trimer (4) is inserted into a so-called precursor complex (5) that comprises the TAP transporter, tapasin, calnexin (CNX) and ERp57. This final fusion yields the mature peptide loading complex (6) and is (according to the standard model) believed to be accompanied by the release of calnexin.

2000; Lindquist et al, 2001) together with the finding that heavy chains are already partially oxidized at this stage (Dick et al, 2004; Tector et al, 1995; Farmery et al, 2000) makes it tempting to speculate that ERp57 is the oxidase, responsible for the formation of the respective disulfide bonds, although a contribution of other ER-resident oxidases as e.g. PDI cannot be excluded. Consistent with this view disulfide-linked dimers of free MHC class I heavy chains and ERp57 could be isolated from human cells (Lindquist et al, 2001) and rat cells expressing human HLA-B27 (Antoniou et al, 2002). Full oxidation of MHC class I heavy chains, which is believed to precede their incorporation into the PLC (Dick et al, 2004) is a prerequisite for an MHC class I molecule to become peptide-receptive as is the association with β_2-microglobulin (Neefjes et al, 1993; Sugita et al, 1994). Binding of β_2-microglobulin to the MHC class I heavy chain is accompanied by the subsequent release of calnexin and thereby dissociates the initial assembly complex (at least in human cells) (Sugita et al, 1994). Since ERp57 on its own has no peptide-binding activity (Ellgaard et al, 2004) and is believed to be recruited to its substrate proteins solely through

interactions with the lectin chaperones it is plausible to assume that ERp57 is also released at this point. Rapidly upon (or concomitantly with) the binding of β_2-microglobulin, calreticulin associates with the assembled MHC class I molecule, which at this stage is likely to harbor monoglucosylated N-linked glycans (Wearsch et al, 2004; Radcliffe et al, 2002). Calreticulin recognizes MHC class I via one of its three exposed sugar trees (Harris et al, 2001). Consistently, treatment with castanospermine, a drug that inhibits the deglucosylation of N-linked glycans prevents the formation of a complex between MHC class I and the lectin chaperone *in vivo* (Sadasivan et al, 1996). In the past it had been postulated that calreticulin tethers ERp57 back into the assembling PLC (Harris et al, 2001). However, this view was challenged by the finding that the oxidoreductase co-precipitates at normal levels with TAP in calreticulin-deficient cells (Gao et al, 2002). So far the exact function that calreticulin exerts within the PLC has not been determined, although diverse roles as e.g. cooperative stabilization of the PLC, chaperoned peptide-delivery from TAP to MHC class I or assisted folding of MHC class I have been proposed (Gao et al, 2002; Culina et al, 2004). One attractive hypothesis postulates that calreticulin tethers empty (or suboptimally loaded) MHC class I molecules that are about to escape the ER back into the PLC (Gao et al, 2002). Such a role in MHC class I retention is in keeping with the observation that cells deficient for calreticulin display accelerated export of unstable, peptide-receptive class I that largely dissociates during (or upon) the migration to the plasma membrane (Gao et al, 2002). It is important to note that the selective retrieval of empty (or suboptimally loaded) MHC class I to the PLC would place calreticulin in the focus of a quality control pathway that ensures the egress from the ER of only highly stable MHC-peptide-complexes. Indeed, this safeguard mechanism is the central function exerted by the PLC (Wright et al, 2004). However, the retention of MHC class I, which is (for

whatever reason) inappropriate for ER export, represents a rather passive contribution to the quality control of peptide loading. A more active role is believed to be played by another chaperone: the 48 kDa transmembrane glycoprotein tapasin. Prior to its incorporation into the PLC tapasin presumably forms a so-called precursor complex that additionally contains the peptide transporter TAP, ERp57 and calnexin (Diedrich et al, 2001). The biogenesis of this early complex is not yet well described. However, a recent report suggests that the assembled PLC finally results from the tapasin-mediated introduction of calreticulin-associated MHC class I into this precursor complex (Diedrich et al, 2001). It was stated that this last step in the assembly of the PLC leads to the release of calnexin (at least in human cells) (Diedrich et al, 2001), so that the PLC in the end consists of TAP, tapasin, calreticulin, ERp57 and MHC class I (Wright et al, 2004). Tapasin binds at the same time MHC class I molecules via its ER-luminal N-terminus (Bangia et al, 1999) and TAP via its C-terminal transmembrane domain (Raghuraman et al, 2002; Lehner et al, 1998; Tan et al, 2002), thereby acting as a bridge between peptide transporter (TAP) and peptide receptor (MHC class I) (Fig. 4). The transmembrane domains of both TAP subunits can bind tapasin independently from each other (Raghuraman et al, 2002). Thus, it was hypothesized that TAP has the potential to form a PLC via TAP1 and TAP2 in a symmetrical manner (Fig. 4). Soon after the discovery of tapasin it was noticed that cells that are deficient for this chaperone display substantially reduced (or complete loss of) MHC class I surface levels, albeit some allelic variation is observed (Garbi et al, 2000; Grandea et al, 1997; Ortmann et al, 1997; Peh et al, 1998). This is a consequence of a series of events that all together have a negative impact on proper loading of MHC class I with optimal high affinity peptide ligands. First, tapasin is required for stabilization of TAP and is therefore needed to maintain high expression levels of the transporter (Garbi et al, 2003;

Lehner et al, 1998; Tan et al, 2002). Additionally, tapasin stimulates the binding of cytosolic peptides to TAP (Li et al, 2000). Thus, in its absence the overall amount of peptides being translocated into the ER is drastically reduced (Abele et al, 2004; Lehner et al, 1998; Li et al, 2000; Momburg et al, 2002). Second, by the above mentioned bridging function a close proximity between TAP and MHC class I is achieved, which may allow for a high local peptide concentration in the immediate vicinity of MHC class I (Abele et al, 2004). In this context, it is important to note that diminished peptide binding by MHC class I in a situation where tapasin is missing has been reported (Dick et al, 2002). It should additionally be mentioned that the close proximity between TAP and MHC class I may ensure that the latter is supplied with that specific pool of peptides that has most recently been generated in the cytosol. The benefit for the immune system could be that viral peptides would efficiently become accessible to class I as early as possible before the antigen processing machinery may be subverted by viral evasion strategies at a later time point during infection (Wright et al, 2004). Third, tapasin is similarly to calreticulin required for the retention of empty or suboptimally loaded class I molecules in the ER (Barnden et al, 2000; Schoenhals et al, 1999). Hence, the absence of tapasin results in a premature release into the secretory route of unstable MHC class I molecules, that later at the cell surface show a drastically increased tendency to acquire exogenously applied peptide (Barnden et al, 2000). Interestingly, one group observed accelerated egress from the ER of MHC class I also in human cells expressing a tapasin variant that fails to interact with TAP (Lehner et al, 1998), pointing towards a role for the TAP-tapasin interaction in the retention of class I. Although, these results are controversially discussed (Tan et al, 2002), they beautifully fit into a model where the PLC acts in the ER-membrane as an anchor that retains yet improperly loaded MHC class I until optimization of its peptide cargo has occurred. In line with this idea

TAP-associated MHC class I displays a dramatically decelerated lateral diffusion within the ER membrane when compared to free peptide-loaded molecules (Marguet et al, 1999). Furthermore, a mutant MHC class I molecule lacking both, tapasin- and calreticulin-interaction was reported to leave the ER loaded with TAP-dependent low-affinity peptides at a highly accelerated rate (Lewis et al, 1996; Lewis et al, 1998; Paulsson et al, 2004; Peace-Brewer et al, 1996). Interestingly, an artificial retention of this MHC class I variant in the ER by transient application of the drug brefeldin A let to a significant improvement in the stability of MHC-peptide complexes (Lewis et al, 1998). This demonstrates that increasing the time that an MHC class I molecule spends in the ER as such has a significant positive effect on its stability and suggests that optimization of peptide cargo is time-dependent. Taken together, it is attractive to speculate that TAP-bound tapasin acts as a docking station in the PLC for calreticulin that functions in the recruitment of empty (or suboptimally loaded) MHC class I molecules for allowing them the acquisition of high affinity ligands. However, the major contribution of tapasin to the quality control of peptide loading does presumably not simply lie in the passive retention of suboptimally loaded MHC class I molecules in the ER. Rather, in the past years a role for tapasin as a peptide editor for MHC class I analogous to the function of the chaperone HLA-DM for MHC class II (Brocke et al, 2002) has been described and is despite one conflicting report (Zarling et al, 2003) widely accepted in the field (Howarth et al, 2004; Sesma et al, 2005; Williams et al, 2002). As a consequence of this peptide editing (or filtering) function exerted by tapasin highly stable MHC-peptide complexes are formed through selective loading of peptide ligands with low off-rates (Howarth et al, 2004; Sesma et al, 2005; Williams et al, 2002). Consistently, the peptide repertoire presented on the surface of tapasin-deficient cells is altered when compared to tapasin-proficient cells (Garbi et al, 2000; Howarth et al, 2004; Purcell et al, 2001; Zarling et al, 2003).

However, the corresponding mechanism is still not clear. A recent review proposes that tapasin deforms the peptide binding pocket of a bound MHC class I molecule in a way that some stabilizing contacts to the peptide cannot be formed (Wright et al, 2004). Under such conditions, which disfavor peptide binding only high affinity ligands are thought to resist the dissociation from MHC class I. It should be noted that this effect may counterbalance the function of tapasin as a facilitator of class I peptide loading (see above) and that some variation with regard to the nature of the peptide ligands would be expected. Further elucidating the role of tapasin in the PLC, several independent studies have shown that tapasin is essential for the recruitment of ERp57 into the complex (Dick et al, 2002; Diedrich et al, 2001; Harris et al, 2001; Hughes et al, 1998; Tan et al, 2002) where both polypeptides are disulfide-linked with each another (Dick et al, 2002). The exact function that the oxidoreductase exerts within the PLC is not known yet. However, in cells expressing a tapasin variant that fails to recruit ERp57 PLC-associated MHC class I molecules were found to be partially reduced (Dick et al, 2002). Crystal structures of class I suggest that the disulfide-bridge in the α2-domain, which is located at the fringe of the peptide binding pocket may be rather exposed (and thereby vulnerable to reduction) in the absence of a peptide ligand (Dick et al, 2004). Therefore, ERp57 may passively shield this disulfide-bridge against reduction by glutathione as long as no (optimal) peptide is bound (Dick et al, 2004). Another model argues in a similar direction by proposing a more active role for ERp57 as an oxidase in the PLC that regenerates partially reduced MHC class I (Dick et al, 2002). ERp57 is further believed to act as a clamp between calreticulin and tapasin, thereby increasing the overall stability of the PLC by cooperative interactions (Wright et al, 2004). Moreover, a periodical resolution of the disulfide-bridge between ERp57 and tapasin was recently proposed to act as a timer for the release of loaded class I from the PLC (Wright et al, 2004). It was

speculated that when this bond is broken the cooperativity of the tapasin-calreticulin-interaction would be disrupted with the consequence that the affinity of tapasin for MHC class I would decrease (Wright et al, 2004). At this stage a bound high affinity peptide could induce the closure of the peptide binding pocket and thereby displace tapasin from class I (Wright et al, 2004). Subsequently, the stably loaded MHC class I molecule could be deglucosylated followed by its release from calreticulin and the PLC (Wright et al, 2004). However, this model is yet speculative and remains to be proven. Another recent report presented evidence that the recruitment of ERp57 to the PLC is essential for the optimization of the peptide cargo loaded onto class I (Dick et al, 2002). Since ERp57 was reported to possess cysteine-protease activity (Urade et al, 1992) this enzyme may directly shape the MHC class I-associated peptide repertoire. However, another study could not validate a role for the oxidoreductase in peptide editing (Howarth et al, 2004). Nevertheless, ERp57 could play a role in the degradation of suboptimally loaded (or empty) MHC class I, thereby helping to selectively remove unstable molecules that otherwise would enter the secretory route. Class I molecules that (for whatever reason) cannot fold or acquire peptide properly are believed to become retrotranslocated into the cytosol, where they are deglycosylated and subsequently degraded by the proteasome (Hughes et al, 1997). Accordingly, inhibitors that interfere with the availability of peptides within the ER like lactacystin (proteasome inhibitor), ALLN (proteasome inhibitor) or ICP47 (TAP inhibitor) induce (or drastically enhance) the transfer of class I back into the cytosol (Hughes et al, 1997), probably using the Sec61 channel (Pilon et al, 1997; Wiertz et al, 1996). The resolution of disulfide-bridges within such molecules is believed to precede their delivery to the cytosol (Tortorella et al, 1998) and may be a prerequisite for the complete unfolding of the protein prior to dislocation. Interestingly, ERp57 was found to possess reductase (in addition to disulfide isomerase and

oxidase) activity *in vitro* (Antoniou et al, 2002; Frickel et al, 2004). Partially folded MHC class I is a substrate for this reductase activity, whereas fully folded molecules are resistant (Antoniou et al, 2002). Although the oxidoreductase does not resolve disulfide bonds within suboptimally loaded MHC class I it acts on peptide-free molecules (Antoniou et al, 2002). Since no biological process works with 100% fidelity, even peptide-loading within the PLC would be expected to yield a small fraction of MHC class I molecules associated with low-affinity ligands. ERp57-mediated reduction of this population after loss of their peptide cargo would prevent the resulting empty class I molecules from binding new peptide outside the PLC and its associated peptide-editing machinery. Regarding this, it is important to note that class I molecules that have dissociated from the PLC are probably deglucosylated (Van Leeuwen et al, 1996) and therefore unlikely to be a substrate for calreticulin-mediated retention. Interestingly, loaded MHC class I molecules were reported to be retained in the ER for a while until export occurs (Marguet et al, 1999). This would provide time for such an additional quality control step. In this context it should be mentioned that calnexin was also reported to be involved in the degradation of glycoproteins. The lectin chaperone was shown to hand over terminally misfolded polypeptides to the acceptor EDEM prior to their retrotranslocation into the cytosol (Molinari et al, 2003; Oda et al, 2003; Sifers et al, 2003). EDEM is an ER stress-inducible membrane protein with homology to α-mannosidase that specifically binds to mannose-N-acetylglucosamine $Man_8GlcNAc_2$ isomer B and has been implicated in the degradation of ER glycoproteins (Molinari et al, 2003; Oda et al, 2003; Sifers et al, 2003). Upon application of proteasome inhibitors a population of MHC class I was reported to redistribute into a subcellular compartment, closely associated with the ER that additionally contains high amounts of calnexin (Kamhi-Nesher et al, 2001). It was speculated that this compartment is involved in the degradation of misfolded

polypeptides (Kamhi-Nesher et al, 2001). The predicted role of ERp57 in the degradation of MHC class I molecules (Antoniou et al, 2002) makes it is tempting to speculate that the oxidoreductase will also localize to the same subregions within the ER. Taken together, it is likely that protein complexes that are associated with MHC class I assembly share a very similar composition with complexes that are involved in MHC class I degradation. Both would be predicted to contain calnexin and ERp57 along with class I.

Interestingly, the amount of PLC-bound ERp57 has been reported to be significantly diminished in the TAP-deficient cell lines .174 (Hughes et al, 1998) and T2 (Momburg et al, 2002) or LCL .220 cells expressing a tapasin variant that fails to associate with TAP (Tan et al, 2002). This shows that TAP is required in human cells for stable integration of ERp57 into the PLC and underscores the importance of cooperative interactions among PLC components for the structural integrity of the complex. Strikingly, the ability of tapasin to bind the peptide transporter was reported to be essential for optimal peptide loading onto MHC class I, but not for high class I surface levels (Lehner et al, 1998; Tan et al, 2002). This becomes evident by an abnormally high thermolability (Gao et al, 2004; Momburg et al, 2002; Tan et al, 2002; Williams et al, 2002) and reduced surface half-life of MHC class I molecules (Tan et al, 2002; Williams et al, 2002) in cells that express tapasin variants, which fail to interact with TAP. However, none of these studies rules out that the low TAP-steady state expression level (as a consequence of lacking transporter stabilization by tapasin (see above)) and therefore the low peptide translocation rate into the ER is the reason for the obvious defect in class I loading. Additionally, given that TAP contributes to the peptide editing process, it is not clear, whether the peptide transporter acts by recruiting ERp57 into the PLC, by cooperatively stabilizing the

PLC, by increasing the local peptide concentration for MHC class I or by other yet unknown mechanisms.

2. Description of the project

Both TAP subunits, TAP1 and TAP2, efficiently associate with tapasin, MHC class I and the accessory chaperones of the PLC when expressed as isolated subunits (Antoniou et al, 2002b; Daumke et al, 2001; Raghuraman et al, 2002). From these studies it had been concluded that cells expressing only one or the other TAP chain can serve as a model system to analyze the protein composition and biogenesis of the PLC. Years ago, it had been postulated that the N-domains that precede the core TMD in both TAP subunits (see above) may act as docking modules for tapasin. This speculation founded on the observation that other ABC proteins as e.g. the sulfonurea receptor (SUR) use their N-terminal extensions to assemble with additional components into higher order complexes (Babenko et al, 2003; Chan et al, 2003). Similarly, the cytosolic N-terminal domain preceding the core TMD in the ABC ion channel CFTR functionally interacts with the SNARE protein syntaxin 1A to regulate the chloride transport (Naren et al, 1998). Thus, to determine whether the N-termini of the TAP chains play a role in the recruitment of tapasin, Koch and co-workers had recently generated a truncation-variant of human TAP, lacking both N-domains. The respective transporter was over-expressed in insect cells either alone or in combination with tapasin. These studies convincingly showed that the mutant transporter could efficiently bind and translocate peptides (Koch et al, 2004), demonstrating that the core TMD is sufficient to allow for the basic functions of the transporter. However, in contrast to wild-type TAP this transporter variant showed no detectable interaction with co-expressed tapasin (Koch et al, 2004), suggesting a key role for the N-domains in the organization of the PLC. In combination with the above mentioned finding that both TAP chains have an intrinsic capability to bind tapasin it was concluded that both N-domains simultaneously serve as tapasin docking sites. However, this conclusion is mainly based on the hypothesis that the interaction of

isolated TAP subunits with tapasin mirrors the respective interactions in the assembled transporter. This hypothesis has never been proven. Moreover, the weak point of the studies undertaken by Koch and co-workers is that they could not analyze the consequences of a possibly deficient PLC formation by N-terminal truncated TAP for MHC class I peptide loading, since they expressed the transporter variant in the absence of class I (Koch et al, 2004). Furthermore, effects of alterations within the PLC on the quality control of peptide loading can anyhow not be satisfactory analyzed in an insect cell system, where MHC class I-mediated antigen presentation does normally not take place. However, these questions are of high significance as in the absence of a proper working quality control instable MHC class I molecules are generated that tend to lose their weakly bound peptide cargo as they migrate along the secretory route. As a consequence they can end up at the cell surface as empty molecules (or even worse they might be reloaded by extracellular peptides and subsequently become recognized by CTLs). Yet, to elucidate the mechanisms that allow the formation of stable MHC class I molecules, presenting high affinity ligands is of fundamental importance to understand how an appropriate cellular immune response against intracellular pathogens is raised.

Hence, to get insight into the function of the N-domains for the loading of MHC class I molecules with peptide ligands, N-terminally truncated TAP chains were expressed in the human TAP-deficient cell line T2 alone, in combination with the corresponding wild-type chain or as head-to-tail fusion proteins. The TAP variants were characterized in different functional assays for their ability to translocate class I ligands across the ER membrane. Moreover, the established cell lines were used to investigate, whether indeed both N-domains of the transporter simultaneously interact with tapasin (see above). Although recent reports have demonstrated that the insertion of TAP into the PLC is essential to allow MHC class I acquiring full

stability (Gao et al, 2004; Momburg et al, 2002; Tan et al, 2002; Williams et al, 2002) the underlying mechanism has not yet been elucidated. Moreover, all studies cited above analyzed cell lines expressing tapasin variants that fully fail to interact with TAP. Thus, it is impossible in the respective experimental systems to distinguish between effects caused by the loss of the TAP1-tapasin-interaction and effects due to the loss of the TAP2-tapasin interaction. In contrast, the cell lines established during this project provided a powerful means to experimentally separate tapasin-binding in the two TAP chains. Thereby they allowed for the first time to investigate the molecular composition and function of PLCs wherein the transporter is incorporated solely via TAP1 or TAP2. Using the respective cell lines it became possible to examine, whether the bridging function of tapasin as such is sufficient to ensure the loading of optimal high affinity ligands as proposed by several groups. In this case bridging of the transporter to MHC class I by either TAP chain would be expected to equally improve the stability of the peptide-loaded molecules. Alternatively, the N-domains of TAP could play a more specific role beyond passively increasing the local peptide concentration for MHC class I during the loading process. To answer the question whether both N-domains are essential for the correct assembly of the PLC and its proper quality control function of MHC class I-loading, the transient association of MHC class I molecules with the PLC, the kinetics of their maturation and finally the characteristics of their surface expression in the established cell lines was investigated. Furthermore, to probe for the stability of intracellular and surface exposed MHC class I molecules in the transfectants, their thermoresistance as well as the time-course of their decay from the cell surface was determined. The findings of these studies demonstrate the important role of the N-domains of TAP for the structural and functional integrity of the PLC. Furthermore, they show that in a situation where the quality control function of the PLC fails, a

post-ER mechanism can rescue the surface expression of stable MHC class I molecules. Thus, the outcome of this work extends the knowledge on the biogenesis of the PLC and provides new insights into the early and late events of antigen presentation.

3. Abbreviations

ADP	adenosine diphosphate
ALLN	N-acetyl-leucyl-leucyl-norleucinal
ATP	adenosine triphosphate
BFA	brefeldin A
BSA	bovine serum albumin
CFTR	cystic fibrosis transmembrane conductance regulator
CNX	calnexin
CR	connector region
CRT	calreticulin
CTL	cytotoxic T-lymphocyte
DMEM	Dulbecco´s modified Eagle´s medium
DTT	1,4-dithio-DL-threitol
ECL	enhanced chemiluminescence
Endo H	endoglycosidase H
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
HBV	hepatitis B virus
HCMV	human cytomegalovirus
HCV	hepatitis C virus
HDA	hexa-D-arginine
HEPES	N-[2-hydroxyethyl]-piperazine-N'-[ethanesulfonic acid]
HIV	human immunodeficiency virus
HKV	influenza A Hong Kong virus
HLA	human leukocyte antigen
HRP	horseradish peroxidase

HRSV	human respiratory syncytial virus
HSAB	N-hydroxysulfosuccinimidyl-4-azidobenzoate
HV68	γ-herpesvirus 68
IMDM	Iscove´s modified Dulbecco´s medium
MDR1	multidrug resistance transporter 1
MFI	mean fluorescence intensity
MHC	major histocompatibility
NBD	nucleotide binding domain
ND	N-domain
NSF	N-ethylmaleimide-sensitive fusion protein
PACE4	paired amino acid converting enzyme 4
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PC	proprotein convertase
PLC	MHC class I peptide loading complex
RPMI	Roswell Park Memorial Institute
SARS	severe acute respiratory syndrome coronavirus
SDS	sodium dodecyl sulphate
SLO	streptolysin O
SNARE	soluble NSF attachment protein receptor
TAP	transporter associated with antigen processing
TBS	Tris-buffered saline
TGN	*trans*-Golgi network
TMD	transmembrane domain
Tris	tris-(hydroxymethyl)-aminomethane
VZV	varicella-zoster virus

4. Materials and Methods

Molecular cloning, polymerase chain reaction (PCR), sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE) and Western blotting were carried out according to standard procedures (Ausubel et al, 1990; Coligan et al, 1995; Sambrook et al, 1989) using enzymes purchased from New England Biolabs, Promega and Amersham. SDS-PAGE was performed under reducing conditions if not otherwise indicated. Immunoblots were developed according to the ECL system (Amersham). Chemicals used were graded p.A. and purchased from Merck or Sigma unless otherwise specified. All solutions and culture media were prepared with ultrapure water derived from a combined reverse osmosis / ultrapure water system (Seral) equipped with UV and ultrafiltration.

4.1 Cell lines and cell culture

T2 is a human lymphoblastoid cell line deficient for the TAP subunits and expresses HLA-A2 and HLA-B5 MHC class I molecules (DeMars et al, 1984; Salter et al, 1985). Transfectants of T2 expressing rat wild-type TAP^a (Momburg et al, 1992) or truncated variants of rat TAP1^a and/or rat TAP2^a were cultured in Iscove´s modified Dulbecco´s medium (IMDM) (Gibco/Invitrogen) supplemented with 10% fetal bovine serum (FBS) and 1 mg/ml G418 (PAA). CEM (Foley et al, 1965) and its calnexin-deficient derivative CEM-NKR (Howell et al, 1985) are T cell leukaemia cell lines that express the MHC class I alleles HLA-A1, HLA-A30, HLA-B21, HLA-Bw6 and HLA-Cw2 (Scott et al, 1995). Both were cultured in IMDM (Gibco/Invitrogen) supplemented with 10% FBS. T1 cells (Salter et al, 1985) were cultured in IMDM (Gibco/Invitrogen) supplemented with 10% FBS. The cervical carcinoma cell line HeLa (Scherer et al, 1953) was grown in IMDM (Gibco/Invitrogen) supplemented with 10% FBS. The monocyte-derived cell line THP-1 (Tsuchiya et al, 1980) was cultured in Roswell Park

Memorial Institute (RPMI) + L-glutamine medium (Gibco/Invitrogen) supplemented with 10% FBS. Murine chemically transformed hepatocytes (TIB-75) (Patek et al, 1978) and the human hepatoma cell line HepG2 (Knowles et al, 1980) were cultured in Dulbecco´s modified Eagle´s medium (DMEM) (Gibco/Invitrogen) supplemented with 10% FBS, 1 mM sodium pyruvate (Gibco/Invitrogen), 2 mM L-glutamine (Gibco/Invitrogen) and non-essential amino acids. All culture media contained 100 U/ml penicillin (Gibco/Invitrogen) and 100 μg/ml streptomycin (Gibco/Invitrogen) to inhibit bacterial growth.

4.2 Cloning and stable expression of TAP chain variants

The vector pBluescript KS II (+) (Stratagene) containing full-length rat TAP1[a] cDNA lacking the internal EcoRI-site (Deverson et al, 1990) or the full-length rat TAP2[a] cDNA (Powis et al, 1991) was used as template in the QuikChange™ site directed mutagenesis procedure (Stratagene) to generate N-terminal truncated variants of TAP1 and TAP2. The complementary primers 5´-GCGCACGCTCGATGCCCTCCGG ACACAAGGGCGCTGG-3´ and 5´-CCAGCGCCCTTGTGTCCGGAGGGCATCGAGC GTGCGC-3´ were used to delete nucleotides 4-417 of rat TAP1[a] (position 1 is the A of the start codon) resulting in a TAP1 variant that lacks the residues 2-139 (1ΔN). Primers 5´-CGCAGACCCCACCATGCCCGGGCAGGAGAATAACAGAGC-3´ and 5´-GCTCTGTTATTCTCCTGCCCGGGCATGGTGGGGTCTGCG-3´ were used to delete nucleotides 4-384 from rat TAP2[a] resulting in a TAP2 variant that lacks the residues 2-128 (2ΔN). All primers were purchased from Invitrogen. From the resulting TAP constructs an EcoRI-fragment was cloned into the EcoRI-site of the expression vector pHßApr1neo (Gunning et al, 1987). The cDNA-constructs of TAP1 and TAP2 were co-transfected in different combinations into TAP-negative T2 cells by

electroporation using a BioRad gene pulser (BioRad) at 270 V and 500 μF. Additionally, cell lines expressing single TAP chain variants were generated. For cloning of the two tandem transporters precursor plasmids were generated in a first step where the sequence encoding either wild-type TAP1 or 1ΔN was followed by the sequence encoding 2ΔN. Therefore, the pBluescript-derivative comprising 2ΔN was digested with ScaI and Acc65I. The resulting 3269 bp-fragment was either ligated to the 4385 bp-fragment from a ScaI/BsrGI-digest of the pBluescript-derivative containing wild-type TAP1 or to the 3971 bp-fragment from a ScaI/BsrGI-digest of the pBluescript-derivative comprising 1ΔN. Both plasmids were digested with AgeI and BbvCI resulting in a 7088 bp- or a 6674 bp-fragment, respectively. The fragments were ligated to the AgeI/BbvCI-fragments containing the linker region of murine MDR1b (Gros et al, 1986) (see below). The generated plasmids encoded tandem transporters containing (TD1/2ΔN) or lacking (TD1ΔN/2ΔN) the N-domain in TAP1. The TAP-tandem variants were cloned as EcoRI-fragments into the expression vector pHβApr1neo (Gunning et al, 1987) and then transfected into T2 cells. The murine MDR1b linker region was cloned by RT-PCR. Total RNA was collected from TIB-75 hepatocytes (Patek et al, 1978) using the RNeasy-Kit (Qiagen). Subsequently, mRNA was prepared using the Oligotex-Kit (Qiagen). An RT-PCR was performed using the SuperScript first-strand synthesis Kit (Invitrogen) in combination with the MDR1b-specific primer 5´-CCCCTACAATCCTTGAAAATACTATGGC-3´ at a concentration of 2 μM. The resulting cDNA served as a template in a standard PCR (the conditions used were identical to that chosen for the determination of the proprotein convertase expression in T2 cells (see below)) , in which primers 5´-CCCCTACAATCCTTGAAAATACTATGGC-3´ and 5´-GGTGGTGTCATTGTGGAGC AAGG-3´ were used to amplify a fragment of 403 bp. This fragment was digested by

SacI and HinP1I yielding a 303 bp-fragment that was ligated with pBluescript KS II (+) cleaved by SacI and AccI. The resulting vector served as a template in a standard Pfu-Polymerase-driven PCR using the TAP-MDR1b-chimeric primers 5´-TGTTACCGGTCCATGGTGGAGGCTCTTGCGGCTCCTTCAGACCCAGGAAATAA TGCTTATGGATCCC-3´ and 5´-ATCCGGCTTGGACAGCCTCAGCAACCGCCAAAA GGAAACCAGAGGC-3´. Thereby a 252 bp-fragment was amplified, which was subsequently digested with AgeI and BbvCI. The resulting 227 bp-fragment encoded the last 12 residues of rat TAP1[a] followed by MDR1b-sequence 640-701 and the residues 140-141 of rat TAP2[a]. This AgeI/BbvCI-fragment was ligated with the AgeI/BbvCI-fragments that were derived from the tandem precursor plasmids (see above). All TAP constructs were fully sequenced from both directions. All cell lines expressing TAP single chains or heterodimeric TAP variants were subcloned. Cells expressing high MHC class I surface levels were enriched by magnetic activated cell sorting (MACS) using the MiniMACS-Separator (Miltenyi Biotec) in combination with the anti-MHC class I-antibody 4E and magnetically labelled rat-anti-mouse IgG2a+b (Miltenyi Biotec). Recovered cells were subcloned for a second time.

4.3 Antibodies

116/5 and D90 are polyclonal rabbit antisera recognizing the C-terminus of rat TAP2 (Momburg et al, 1992) or rat TAP1 (Powis et al, 1991). 148.3 is a monoclonal antibody directed against the C-terminus of human TAP1 (Meyer et al, 1994). 3B10.7 is a monoclonal rat anti-human MHC class I antibody binding the heavy chains of HLA-A and -B (Lutz et al, 1987). 4E is a β_2m-dependent mouse monoclonal antibody that recognizes an epitope common to all assembled HLA-B molecules (Yang et al, 1984; Trapani et al, 1989; Tector et al, 1995). w6/32 is a mouse monoclonal antibody

that recognizes β_2m-associated HLA-A, HLA-B and HLA-C molecules (Barnstable et al, 1978). Anti-ER60 is a rabbit antiserum that binds the oxidoreductase ERp57 (Otsu et al, 1995). R.gp48N is a polyclonal rabbit antiserum that is specific for the N-terminus of human tapasin (Sadasivan et al, 1996). SPA-600 and -865 are polyclonal rabbit antisera binding calreticulin or calnexin (StressGen). MP-1 and KP-1 are polyclonal antisera, directed against the N-terminus or the C-terminus of the proprotein convertase 7 (PC7), respectively (Van de Loo et al, 1997). Anti-furin is a polyclonal antiserum raised against the proprotein convertase furin (Leitlein et al, 2001). MAC 256 is a rat-derived monoclonal antibody recognizing the ER retention motif KDEL (Napier et al, 1992). Alexa-488 labeled donkey-anti-rat IgG and Alexa-546 labeled goat-anti-rabbit IgG were purchased from Molecular Probes. Fluorescein isothiocyanate (FITC)- and horseradish peroxidase (HRP)-conjugated antibodies were purchased from Dianova or Amersham.

4.4 Immunoprecipitation and Western blot analysis

1 x 10^7 cells were washed twice in PBS before lysis in PBS / 1% Triton-X-100 (Sigma). In case that the intact PLC should be co-isolated lysis was performed in PBS / 1% digitonin (Wako) as this detergent is known to preserve the TAP-tapasin association. Postnuclear supernatants were subsequently applied for at least 16 hours at 4 °C to the respective antibody linked to sepharose beads for allowing immunoadsorption to occur. Antibodies had been covalently coupled before either to cyanogen bromide-activated sepharose (Sigma) in case that anti-MHC class I antibodies 4E or 3B10.7 were used or protein A-sepharose (Amersham) in case that anti-TAP1 antibodies 148.3 or D90 or the anti-TAP2 antiserum 116/5 was used. The coupling procedure was performed according to the instructions of the manufacturer. After extensive washing of the precipitate with PBS / 1% Triton-X-100 or PBS / 0,1%

digitonin (depending on the detergent used for lysis) bound proteins were eluted in 100 mM Tris / 0,5% SDS pH 9,0. In case that consecutive immunoprecipitation sequentially using the anti-TAP2 antiserum 116/5 followed by the monoclonal antibody 3B10.7 was performed, bound proteins were eluted from the 116/5-sepharose by a 2 hour incubation at room temperature in the presence of 100 μM of a peptide corresponding to the C-terminus of rat TAP2 (EQDVYAHLVQQRLEA, in single letter amino acid code) (Momburg et al, 1992) diluted in PBS / 1% digitonin. Eluted samples were separated by SDS-PAGE under reducing conditions. In case that calreticulin should be detected later on, SDS-PAGE was performed under non-reducing conditions. Separated proteins were transferred to a nitrocellulose membrane (Schleicher & Schuell) before blocking in PBS / 0,1% Tween20 / 5% dry milk powder for 16 hours at 4 °C. Specific polypeptides were decorated with the respective primary antibodies diluted in PBS / 10% FBS / 0,1% Tween20 followed by extensive washing using PBS / 0,1% Tween20. This was followed by incubation of the membrane with HRP-conjugated secondary antibody. Unspecifically bound antibody was removed by washing in PBS / 0,3% Tween20. Finally immunoblots were developed according to the ECL system (Amersham).

4.5 Inhibition of proteasomal activity with ALLN

T2 transfectants were incubated in the absence or presence of 250 μM ALLN (Calbiochem) for 4h at 37 °C. Subsequently, cells were lysed in PBS / 1% digitonin either immediately or after removal of the inhibitor by extensive washing followed by overnight incubation at 37 °C in inhibitor-free IMDM containing 10% FBS. TAP-associated proteins were isolated using the TAP2-specific antibody 116/5. Where indicated, MHC class I-associated proteins were re-precipitated from the eluate. Isolated proteins were separated by SDS-PAGE and analyzed by Western blot.

4.6 Pulse-chase analysis

Transfectants were starved for 2h in methionine/cysteine-free Roswell Park Memorial Institute (RPMI) medium. Then, [^{35}S]methionine and [^{35}S]cysteine (500 µCi Promix/ml, Amersham) was added for 45 min. The chase was initiated by the addition of medium containing a 10-fold excess of unlabelled methionine and cysteine. Equal numbers of cells were removed at various times of chase and washed in phosphate-buffered saline (PBS) before resuspension in lysis buffer (PBS containing 1% digitonin (Wako) or 1% Triton X-100 (Sigma)). Immunoprecipitations were performed from equivalent amounts of precleared lysates by using anti-TAP2 antiserum (116/5) or anti-MHC class I antibody (4E). The samples were washed five times with lysis buffer and subsequently separated by SDS-PAGE under reducing conditions. The gels were stained with Coomassie brilliant blue (Roth) and dried. Fluorographs were obtained after different exposure times and scanned by microdensitometry using a Joyce-Loebl Chromoscan II (Joyce-Loebl).

4.7 Determination of MHC class I thermostability in detergent extracts

1 x 10^8 cells were washed twice with ice-cold PBS before lysis in PBS containing 1% NP-40 (Sigma) and protease inhibitor-cocktail (Roche). When indicated, lysates were pre-incubated for 1 hour with 20 µM of HLA-B5 binding peptide EBV-3C/881-9 (QPRAPIRPI, in single letter amino acid code) (Hill et al, 1995) at 4°C. Subsequently, lysates were incubated for 30 min at 4°C (where suboptimally loaded or empty class I molecules are preserved) or at 37°C (where only stable class I molecules resist dissociation). Afterwards lysates were immunoprecipitated using the monoclonal anti-MHC class I antibodies 4E (conformation-dependent) or 3B10.7 (conformation-independent). Precipitates were analyzed by Western blot and the obtained signals were quantified by densitometric scanning.

4.8 Immunofluorescence

5 x 10^6 cells were washed twice with PBS before fixation in PBS containing 3% paraformaldehyde for 15 minutes. Subsequently cells were extensively washed with PBS / 10 mM glycine before resuspension in PBS / 0,5 % BSA. Next, the cells were permeabilized in PBS containing 0,5 % BSA and 0,5 % saponin and additionally a combination of anti-TAP antibody (either D90 directed against TAP1 or 116/5 directed against TAP2, both of rabbit origin) and anti-KDEL antibody (MAC 256, derived from rat) was applied. After 30 minutes incubation, cells were washed twice with PBS / 0,5 % BSA / 0,5 % saponin before addition of a combination of two secondary antibodies, namely Alexa 488-coupled anti-rat IgG and Alexa 546-coupled anti-rabbit IgG. Unspecifically bound antibody was removed by washing with PBS / 0,5 % BSA / 0,5 % saponin. Subsequently, cells were resuspended in 20 µl mowiol and transferred to an object slide. Stained cells were analyzed using a Zeiss Axioplan II fluorescence microscope equipped with a cooled CCD camera (Quantix).

4.9 Deglycosylation of MHC class I molecules with endoglycosidase H (EndoH)

10 µl of a Triton X-100 lysate, corresponding to 1 x 10^5 cells was added to 20 µl EndoH-incubation buffer (50 mM trisodium citrate / 0,01% SDS / 0,1 M 2-mercaptoethanol) and mildly vortexed for 30 minutes before addition of 7,5 mU endoglycosidase H (Roche). Digestion was performed at 37 °C for 16 hours and the glycosylation pattern of MHC class I molecules, which corresponds to the steady state distribution of MHC class I molecules between the ER and post-ER compartments was determined by Western blot, using the monoclonal antibody 3B10.7. The relative intensity of the bands was quantified by densitometric scanning, using the Joyce-Loebl Chromoscan II densitometer (Joyce-Loebl).

4.10 Flow cytometry and determination of MHC class I thermostability in intact cells

5 x 10^6 cells were washed twice in ice-cold DFN (PBS / 10% FBS / 0,9 mM $CaCl_2$*$2H_2O$ / 0,5 mM $MgCl_2$*$6H_2O$ / 0,01% NaN_3) and subsequently stained with monoclonal HLA-B5-reactive antibody 4E diluted in DFN at a final concentration of 100 μg/ml for 30 min at 4 °C. After four-fold washing with ice-cold DFN cells were incubated with secondary FITC-labelled anti-mouse IgG antibody for 30 min at 4 °C in the dark. After four-fold washing with ice-cold DFN 3 μl propidium iodide (Sigma) was added to stain permeabilized (dead) cells. Subsequently, HLA-B5 surface levels were determined using a FACScan flow cytometer (Becton-Dickinson). To investigate the heat-sensitivity of intracellular and surface MHC class I, transfectants were incubated for 10 min at 4, 40, 50 and 60°C. Thermostability of intracellular HLA-B5 was analyzed by an experiment in which cells were treated with 0.5% paraformaldehyde for 15 min and washed with 10 mM glycine. Cells were permeabilized in PBS containing 0.5% saponin / 0.5% BSA. Simultaneously the monoclonal antibody 4E was applied for 30 min at room temperature. Before addition of FITC-coupled anti-mouse IgG unspecifically bound antibodies were removed by washing with PBS containing 0.5% saponin and 0.5% BSA. The thermostability of MHC class I molecules in CEM or CEM-NKR cells was examined in an analogous procedure with the exception that the monoclonal antibody w6/32 was used instead of the antibody 4E. To determine thermostability of surface HLA-B5, cells were directly immunostained with mAb 4E without fixation and permeabilization. The dependence of MHC class I presentation on endocytic recycling and endosomal processing was analyzed by an experiment in which cells were grown in IMDM containing 250 μM primaquine or 20 mM NH_4Cl (Sigma) for 3 and 18h, respectively. To determine the surface-survival of HLA-B5 cells were incubated for 0h, 1h, 2h, 4h,

12h or 15h in the presence of brefeldin A (BFA) (Sigma) at a final concentration of 10 ng/μl. To analyse the involvement of proprotein convertases (PCs) in antigen presentation cells were washed with FBS-free IMDM followed by removal of surface MHC class I molecules by incubation with IMDM / 50 mM Na-citrate pH 3.0 for 3 min. After neutralizing the pH with 150 mM NaH_2PO_4, pH 10.5 recovery of surface MHC class I was allowed to occur. Therefore, the transfectants were washed and incubated for 14 h at 37°C in FBS-free IMDM with or without 10, 33 or 100 μM hexa-D-arginine (HDA) (Calbiochem).

4.11 Nucleotide-binding assay

2 x 10^7 cells were washed twice with ice-cold Tris-buffered saline (TBS) (50 mM Tris / 150 mM NaCl pH 7,4) comprising 5 mM $MgCl_2$ followed by a short incubation in 10 mM Tris pH 7,4 (hypotonic buffer) at 4 °C. Subsequently, the cells were homogenized and nuclei were removed by centrifugation. The postnuclear supernatants were subjected to ultracentrifugation for the isolation of endomembranes, which were lysed for 1 hour at 4 °C in TBS / 5 mM $MgCl_2$ / 1% Triton-X-100. Lysed membranes were again centrifuged and precipitated (insoluble) material was discarded. The supernatant was applied either to ATP- or ADP-agarose (both purchased from Sigma), which had been equilibrated according to the instructions of the manufacturer. After 1 hour incubation at 4 °C precipitated material was washed 5 times with TBS / 5 mM $MgCl_2$ / 1% Triton X-100, eluted in SDS-containing sample buffer and analyzed in Western blots probed for TAP1 or TAP2.

4.12 Radioiodination of peptides

15 nmol of peptide S5 (TVDNKTRYV, in single letter amino acid code) (Momburg et al, 1994) or S8 (TVDNKTRYR, in single letter amino acid code) (Deverson et al,

1998) were suspended in 100 mM phosphate buffer pH 7,5 and subsequently mixed with 5 µl of a 74 MBq solution of the radioisotope ^{125}I (Amersham). To radioiodinate tyrosine residues Chloramin T-solution (Sigma) was added. Hereafter, free iodine was removed using a Dowex ion exchange column (Supelco) and the labelled peptide was eluted in phosphate buffer pH 7,5.

4.13 Peptide cross-linking

2 x 10^6 cells were permeabilized for 15 min at 37 °C using streptolysin O (SLO) (Murex) at a concentration of 2 U / ml. After washing with ice-cold PBS cells were resuspended in peptide binding buffer (50 mM Tris / 1 mM $CaCl_2$ / 5 mM $Mg(OAc)_2$ / 1 mM DTT (Sigma) / 0,1% dialyzed denatured BSA / protease inhibitor-cocktail (Roche) pH 7,5). Subsequently, 1 µM radioiodinated and HSAB-conjugated peptide S5 (TVDNKTRYV, in single letter amino acid code) (Momburg et al, 1994) or S8 (TVDNKTRYR, in single letter amino acid code) (Deverson et al, 1998) was applied, followed by UV-mediated (254 nm) cross-linking of bound peptide to TAP for 5 min on ice. Hereafter, cells were lysed in PBS / 1% NP-40 (Sigma) and the postnuclear supernatants were subjected to immunoprecipitation using the anti-TAP2 antibody 116/5. Precipitated proteins were separated by SDS-PAGE. The gels were stained with Coomassie brilliant blue (Roth), dried and subsequently analyzed by autoradiography.

4.14 Peptide transport assay

3,75 x 10^6 cells were permeabilized for 15 min at 37 °C using streptolysin O (SLO) (Murex) at a concentration of 2 U / ml. Following a short wash with ice-cold PBS cells were resuspended in peptide transport buffer (50 mM HEPES / 150 mM KOAc / 5 mM $Mg(OAc)_2$ / 250 mM D(+)-sucrose / 1 mM DTT / protease inhibitor-cocktail

(Roche) pH 7,5) in the presence of 10 mM ATP. Radioiodinated peptide S5 (TVDNKTRYV, in single letter amino acid code) (Momburg et al, 1994) or S8 (TVDNKTRYR, in single letter amino acid code) (Deverson et al, 1998) was added to a final concentration of 1,5 μM and subsequently the cells were incubated at 37 °C for 15 min to allow for the transport of the labelled peptide into the ER (, which is resistant to permeabilization by SLO). Hereafter, cells were lysed for 5 min at room temperature in ConcanavalinA (ConA)-binding buffer (20 mM Tris / 500 mM NaCl / 1 mM $CaCl_2$ / 1 mM $Mn(II)Cl_2$ / 0,1% Triton X100 / 0,1% NP-40 / protease inhibitor-cocktail (Roche) pH 7,5). Postnuclear supernatants were applied to ConA-sepharose (Sigma) and binding of glycosylated (transported peptides) was allowed to occur for 1 hour. Subsequently, the precipitates were washed 5 times with ConA-binding buffer and recovered radioactivity was quantitated by γ-counting. To directly compare the transport activities of wild-type TAP and truncated TAP variants the results of the transport assays were normalized for the expression levels of the different transporters. For quantitation of TAP steady-state levels, equivalent numbers of cells were lysed in 1% Triton X-100. Lysates were serially diluted 2-fold and separated by SDS-PAGE. After protein transfer onto nitrocellulose immunoblots were probed for TAP1 and TAP2. Quantification by densitometric scanning revealed very similar steady-state expression levels for TAPwt, 1-2ΔN and 2-1ΔN, but significantly lower expression levels for the tandem transporters TD1/2ΔN and TD1ΔN/2ΔN.

4.15 Determination of proprotein convertase expression by RT-PCR

mRNA isolated from the T2 cell line as well as from the HepG2 cell line was kindly provided by Cemalettin Bekpen. Using this mRNA as a template the SuperScript first-strand synthesis Kit (Invitrogen) in combination with 2 μM of a standard oligo(dT)-

primer (Invitrogen) was employed to perform RT-PCR. The resulting cDNA served as a template in a standard PCR to probe for the expression of individual proprotein convertases. All primer pairs were designed so that the amplified region is a part of all known isoforms of the respective gene and that it contains a large intron to exclude that a contamination by genomic DNA rather than cDNA served as a template for the PCR. The primer pair 5´-GCAACGGAGGCCAACACAACG-3´ and 5´-GCGTTGAGGAGGCCGAAACCG-3´ was used to amplify a 424 bp-fragment from the cDNA, corresponding to the PC7 gene (Bruzzaniti et al, 1996). The primer pair 5´-CTACGGCACCTCCGTGCAGCC-3´ and 5´-CTTGAATCCTGGGCCATTGCACG-3´ was used to amplify a 769 bp-fragment from the cDNA, corresponding to the PC5 gene (Mercure et al, 1996). The primer pair 5´-CAAACAATTCCTACTGCATCGTGG GC-3´ and 5´-CCGTTCACTTTCCAGTCGCTC GC-3´ was used to amplify a 628 bp-fragment from the cDNA, corresponding to the PACE4 gene (Kiefer et al, 1991). The primer pair 5´-CGAGGATGACGGCAAGACAGTGG-3´ and 5´-CGGCGATTATAGGA CAGGGTGAGC-3´ was used to amplify a 726 bp-fragment from the cDNA, corresponding to the furin gene (Roebroek et al, 1986). The DNA was amplified under the following conditions: 94 °C for 2 min; 39 cycles of 94 °C for 30s, 65 °C for 30s and 72 °C for 1 min; and a final extension at 72 °C for 15 min. The PCR products were analyzed on a 2% agarose gel.

4.16 Examination of proprotein convertase regulation by interferons

The cell lines T2, HeLa or THP-1 were treated for 24 hours with 200 U/ml interferon-γ (Peprotech) or 2000 U/ml interferon-β (PBL Biomedical Laboratories) before mRNA was prepared by the sequential use of the RNeasy-Kit (Qiagen) (for isolation of total RNA) and the Oligotex-Kit (Qiagen) (for isolation of mRNA). The resulting mRNA, which was kindly provided by C. Bekpen served as a template for cDNA synthesis by

RT-PCR using the SuperScript first-strand synthesis Kit (Invitrogen) in combination with 2 μM of a standard oligo(dT)-primer (Invitrogen). This cDNA was used as a template in a standard PCR to probe for the expression of the indicated genes. The primer pair 5´-CGAGGATGACGGCAAGACAGTGG-3´ and 5´-CGGCGATTATAGGA CAGGGTGAGC-3´ was used to amplify a 726 bp-fragment from the cDNA, corresponding to the furin gene (Roebroek et al, 1986). The primer pair 5´-GCAACG GAGGCCAACACAACG-3´ and 5´-GCGTTGAGGAGGCCGAAACCG-3´ was used to amplify a 424 bp-fragment from the cDNA, corresponding to the PC7 gene (Bruzzaniti et al, 1996). The primer pair 5´-CAAACAATTCCTACTGCATCGTGGGC-3´ and 5´-CCGTTCACTTTCCAGTCGCTCGC-3´ was used to amplify a 628 bp-fragment from the cDNA, corresponding to the PACE4 gene (Kiefer et al, 1991). The primer pair 5´-CTGAGCCTGTCCGAAGCCCTGC-3´ and 5´-GGACTTGGCGGTTCT GTGGAGG-3´ was used to amplify a 645 bp-fragment from the cDNA corresponding to the MxA gene (Aebi et al, 1989). The primer pair 5´-ATGACAACTTTGGTATCG TGGAAGG-3´ and 5´-GAAATGAGCTTGACAAAGTGGTCGT-3´ was used to amplify a 442 bp-fragment from the cDNA corresponding to the GAPDH gene (Hanauer et al, 1984). The primer pair 5´-ATCTGGATGTGGTGTGTGCC-3´ and 5´-CTTGTAGTTTC TCGAGCTGG-3´ was used to amplify a 532 bp-fragment from the cDNA corresponding to the hGBP1 gene (Cheng et al, 1991). PCR was performed as described in the section above with modifications on the cycle number and the hybridization temperature. These were 25 cycles with a hybridization temperature of 60 °C in the case that fragments corresponding to GAPDH, hGBP1 or MxA were amplified and 31 cycles with a hybridization temperature of 65 °C in the case that fragments corresponding to furin, PC7 or PACE4 were amplified. The PCR products were analyzed on a 2% agarose gel.

4.17 Preparation of microsomes from T2 cells by sucrose gradient fractionation

1 x 10^8 cells were incubated in ice-cold 10 mM Tris pH 7,5 (hypotonic buffer) for 10 min. Hereafter, the cells were homogenized and the nuclei were removed by centrifugation at 1600g and 4 °C. 0,33 vol 1,3 M sucrose buffer (20 mM HEPES pH 7,5 / 25 mM CH_3COOK / 5 mM $(CH_3COO)_2Mg*4H_2O$ / 1 mM DTT / protease inhibitor-cocktail (Roche)) were added to the supernatant, which was again centrifuged at 1600g and 4 °C. Following this, the postnuclear supernatant was subjected to ultracentrifugation at 100000g and 4 °C to isolate endomembranes, which were subsequently resuspended in 800 µl 0,25 M sucrose buffer. Next, 7 vol 2,5 M sucrose buffer were added and the solution was mixed for 1 hour at 4 °C. Hereafter, the membrane suspension was overlaid with 3 ml 2 M and 3 ml 1,3 M sucrose buffer. Finally, 800 µl 0,25 M sucrose were loaded onto the top of the gradient. To fractionate the membranes the sucrose gradient was centrifuged for 16 h at 4 °C (25500 rpm / SW41Ti-rotor / Beckman). The microsomes were subsequently collected at the interface between the1,3 M and 2 M sucrose buffer. After the addition of 1 vol 20 mM HEPES buffer (20 mM HEPES pH 7,5 / 25 mM CH_3COOK / 5 mM $(CH_3COO)_2Mg*4H_2O$ / 1 mM DTT / protease inhibitor-cocktail (Roche)) the microsomal suspension was centrifuged at 100000g and 4 °C, resuspended in 400 µl membrane buffer (50 mM HEPES pH 7,5 / 250 mM sucrose / 1 mM DTT / protease inhibitor-cocktail (Roche)) and homogenized. Subsequently, 10 vol high salt buffer (50 mM HEPES pH 7,5 / 250 mM sucrose / 1 M KCl / 10 mM $MgCl_2*6H_2O$ / 1 mM DTT / protease inhibitor-cocktail (Roche)) were added and the suspension was again centrifuged at 100000g and 4 °C. Following this, the microsomal pellet was washed with 1 ml high salt buffer and afterwards resuspended in membrane buffer at a final concentration of 1 equivalent/µl. Subsequently, the microsomes were again homogenized, snap frozen in liquid nitrogen and stored at -80 °C.

4.18 *In vitro* translation

The TNT SP6 Quick Coupled Transcription/Translation System (Promega) was used to *in* vitro-express TAP2 variants in microsomal membranes. The pSP64 vector (Promega) containing wild-type TAP2 (pSP64-TAP2) was kindly provided by K. Keusekotten. A pSP64-derivative encoding 2ΔN was generated by site directed mutagenesis using the QuikChange™ protocol (Stratagene) in combination with the primer pair 5´-CGCAGACCCCACCATGGCGCCCGGGCAGG-3´ and 5´-CCTGCCC GGGCGCCATGGTGGGGTCTGCG-3´ and pSP64-TAP2 as a template. For *in vitro*-expression 20 μl of microsomes (1 equivalent/μl) were pre-incubated with 0,5 μl RNasin-ribonuclease inhibitor (40U/μl) (Promega) in a first step. Furthermore, also 5 μl plasmid DNA (500 ng/μl), encoding either TAP2 or 2ΔN were pre-incubated with 0,5 μl RNasin-ribonuclease inhibitor (40U/μl) (Promega). Subsequently, 40 μl of the TNT Quick Master Mix (Promega), 3 equivalents of microsomes, 500 ng plasmid DNA, 2 μl Diethylpyrocarbonate (DEPC)-H_2O were incubated at 30 °C for 90 min in the presence of 80 μM methionine (Promega) in a final volume of 50 μl.

4.19 Immunodepletion

Following the *in vitro*-translation of mutant or wild-type TAP2 chains into microsomes derived from T2 transfectants, the membranes were collected by centrifugation at 100000g and 4 °C and washed twice with PBS. Subsequently, the microsomes were solubilized in PBS / 1% digitonin (Wako). The newly synthesized TAP2 chains were removed from the resulting lysate in two successive rounds of immunodepletion using protein A-sepharose-conjugated anti-TAP2 antibodies (116/5). To control for the specificity of the immunodepletion microsomal lysates were incubated with free protein A-sepharose (Amersham) in parallel. To examine the heterodimerization of

the TAP subunits, the depleted lysates were analyzed in Western blots probed for TAP1 and TAP2.

5. Results

5.1 ER-export and surface expression of MHC class I does not depend on the number or position of tapasin-docking sites in TAP

To investigate the function of the N-domains in TAP for MHC class I processing, N-terminally truncated variants of both rat TAP[a] subunits were constructed (see section 4.2). The resulting mutant TAP chains, lacking the residues 2-139 in rat TAP1[a] or the residues 2-128 in rat TAP2[a] were named 1ΔN or 2ΔN, respectively (Fig. 5A). Both TAP chain variants were stably co-transfected or transfected in combination with the corresponding wild-type chain into the TAP-negative human cell line T2. Transporters containing wild-type TAP1 and 2ΔN were termed 1-2ΔN while transporters consisting of wild-type TAP2 and 1ΔN were named 2-1ΔN. Surprisingly, transfectants co-expressing 1ΔN and 2ΔN could not be established, although more than 100 different clones from four independent transfection experiments were analyzed (data not

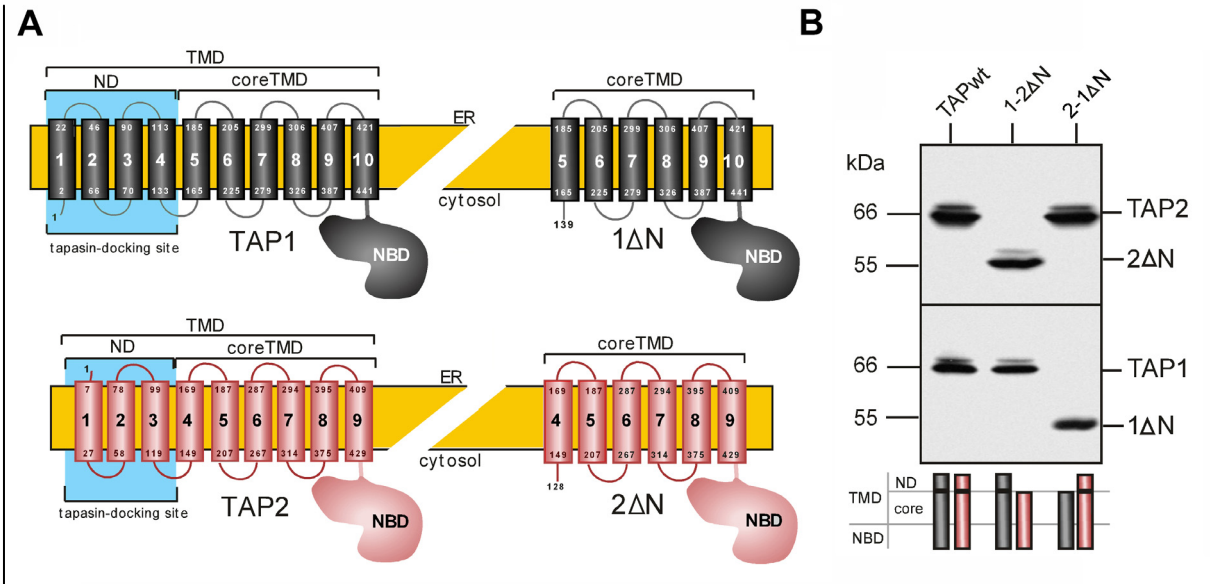

Fig. 5 **Expression of TAP variants lacking the N-domain in TAP1 or TAP2**
A) Schematic diagrams of wild-type TAP subunits, 1ΔN and 2ΔN. N-domains (ND), core transmembrane domains (core TMD), transmembrane segments and nucleotide binding domains (NBD) are indicated. Topology prediction of the TMDs was performed with TopPred II (Claros et al, 1994). The regions corresponding to the tapasin-docking sites (Koch et al, 2004) are indicated. **B)** Expression of 1-2ΔN and 2-1ΔN. Cell lysates were analyzed in Western blots probed for TAP1 or TAP2. TAP1 (black) and TAP2 (red) are shown as pictograms.

shown). This suggests that stable expression of a heterodimeric transporter variant lacking both N-domains is either harmful to the transfected T2 cell and/or that the TAP variant is structurally unstable and rapidly degraded. Nevertheless, Fig. 5B shows that 1-2ΔN and 2-1ΔN were expressed at levels comparable to that of the wild-type transporter. To functionally characterize 1-2ΔN and 2-1ΔN, the peptide-binding behaviour of the two TAP variants was determined in a first step (Fig. 6A).

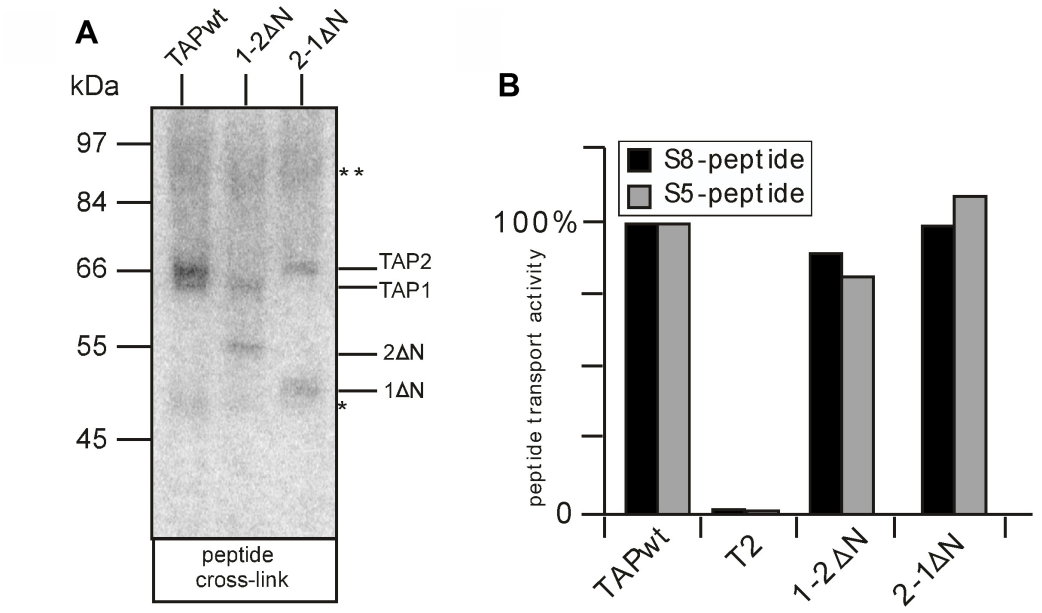

Fig. 6 **Peptide-binding and transport activity of TAP truncation variants**
A) Peptide-binding of TAPwt, 1-2ΔN and 2-1ΔN. Permeabilized cells were incubated with radiolabeled and HSAB-conjugated peptide S8 (TVDNKTRYR). After UV-cross-linking and membrane lysis, TAP was immunoisolated with αTAP2 antiserum. Asterisks indicate unidentified cross-link products. **B)** Peptide transport activity. Cells were permeabilized and incubated with radiolabeled peptides S5 (TVDNKTRYV) or S8 (TVDNKTRYR) in the presence of ATP. The transport activity measured for 1-2ΔN and 2-1ΔN is expressed as percentage of the transport activity (quantified by γ-counting) obtained for TAPwt.

Therefore, photolabeling experiments were carried out using the radioiodinated, UV-cross-linkable model peptide S8 (TVDNKTRYR, in single letter amino acid code) (Deverson et al, 1998). Similar to wild-type TAP, 1-2ΔN and 2-1ΔN showed peptide-cross-linking to both transporter subunits (Fig. 6A) suggesting normal substrate uptake by the truncated TAP variants. Next, the peptide translocation activity of 1-2ΔN and 2-1ΔN was measured (Fig. 6B) using the model peptides S5 (TVDNKTRYV, in single letter amino acid code) (Momburg et al, 1994) or S8 (TVDNKTRYR, in single

letter amino acid code) (Deverson et al, 1998). Consistent with the previous observation that the N-domains are not required for the transport function of TAP (Koch et al, 2004), both variants showed peptide-translocation-activities within the range of the wild-type transporter (Fig. 6B).

Based on biochemical and structural data regarding the TAP-related ABC-transporters MDR1 (Loo et al, 2004) and MsbA (Chang et al, 2001) it is plausible to speculate that the N-domains of TAP1 and TAP2 point in the heterodimeric transporter to opposite sides of the paired core TMDs (Fig. 2B) and act as two autonomous tapasin-docking sites for the transient interaction with MHC class I molecules. To investigate this, the ability of the truncated transporter variants to interact with tapasin and MHC class I was determined. Therefore, TAP or MHC class I molecules were immunoisolated from digitonin lysates of the cell lines T2(TAPwt), T2(1-2ΔN) or T2(2-1ΔN). Co-isolated proteins were subsequently analyzed by Western blot (Fig. 7A and Fig. 16). Clearly, both truncated transporter variants retained the ability to form complexes with tapasin and MHC class I. However, the anti-TAP immunoprecipitations revealed that the tapasin-interaction is reduced in the truncated TAP variants by approx. 50% when compared to the wild-type transporter, indicating that the latter binds tapasin via both N-domains. In contrast, the amounts of MHC class I co-precipitated with TAP were almost identical for the different transporter variants, indicating that the MHC class I-interaction is comparable among TAPwt, 1-2ΔN and 2-1ΔN. In accordance with this, pulse-chase experiments (Fig. 7B) revealed that the half-life of interaction between newly synthesized MHC class I and the TAP-containing PLC is identical (approx. 100 min), irrespective whether the latter comprises TAPwt, 1-2ΔN or 2-1ΔN. Thus, despite reduced tapasin-binding, the PLCs formed by 1-2ΔN and 2-1ΔN show a normal transient interaction with peptide-receptive MHC class I molecules.

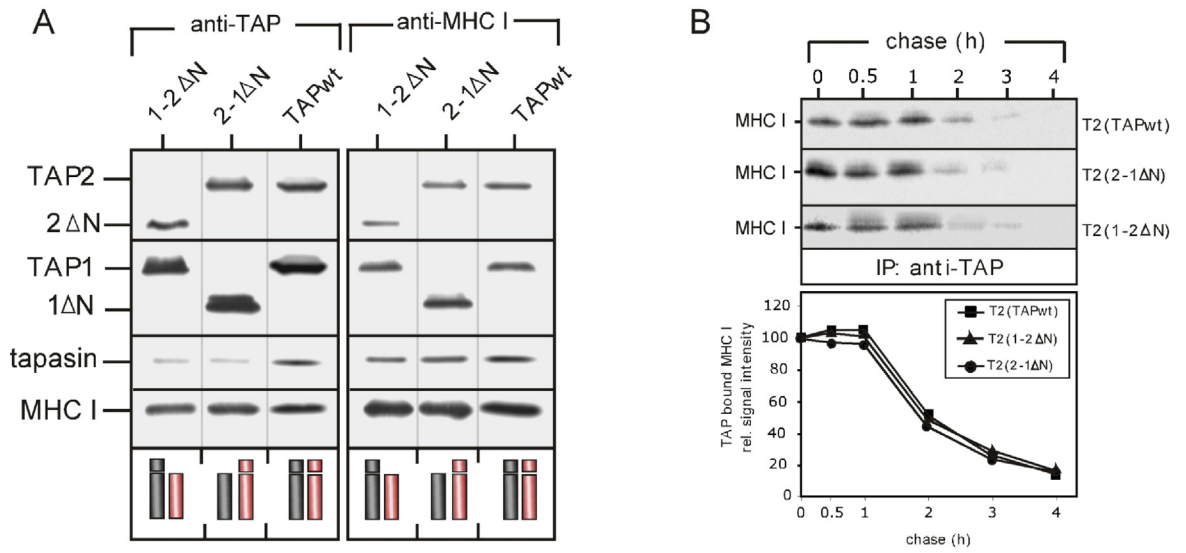

Fig. 7 **Functional characterization of PLCs that comprise TAP variants, which lack the N-domain in TAP1 or TAP2.**
A) TAP/tapasin-interaction. Digitonin lysates of transfectants expressing truncated or wild-type TAPs were subjected to immunoprecipitation using the αTAP2 antiserum 116/5 or the monoclonal αMHC class I antibody 3B10.7. Precipitates were analyzed in Western blots probed for TAP1, TAP2, tapasin or MHC class I heavy chains. TAP1 (black) and TAP2 (red) are shown as pictograms. **B)** Kinetics of TAP-MHC class I interaction in different transfectants. Cells were pulse-labeled for 60 min. and chased for the indicated time points. Lysates from equal numbers of cells were immunoprecipitated with αTAP2 antiserum and resolved by SDS-PAGE. Quantification of the co-isolated MHC class I signals was performed by densitometric scanning of the fluorographs. The amount of TAP-associated MHC class I isolated at each time point is expressed as percentage of the respective amounts detected directly after the pulse.

In further approaches the intracellular processing (Fig. 8A), the kinetics of transport to the cell surface (Fig. 8B) and the steady-state surface expression (Fig. 8C) of MHC class I in the different transfectants was analyzed. Endoglycosidase H (endo H)-assays carried out using cell extracts from T2(TAPwt), T2(1-2ΔN) and T2(2-1ΔN) revealed that under steady-state conditions about 80% of MHC class I is present in post-ER compartments whereas in non-transfected T2 cells more than 80% of MHC class I is retained in the ER (Fig. 8A). In accordance with this, the recovery of HLA-B5 surface levels after removal of extracellularly exposed MHC class I molecules through a short application of acidic buffer was similar in cell lines expressing TAPwt, 1-2ΔN and 2-1ΔN (Fig. 8B). Consequently, the steady-state surface HLA-B5 levels in the transfectants T2(1-2ΔN) and T2(2-1ΔN) were determined to be almost identical to that observed in the cell line T2(TAPwt) (Fig. 8C).

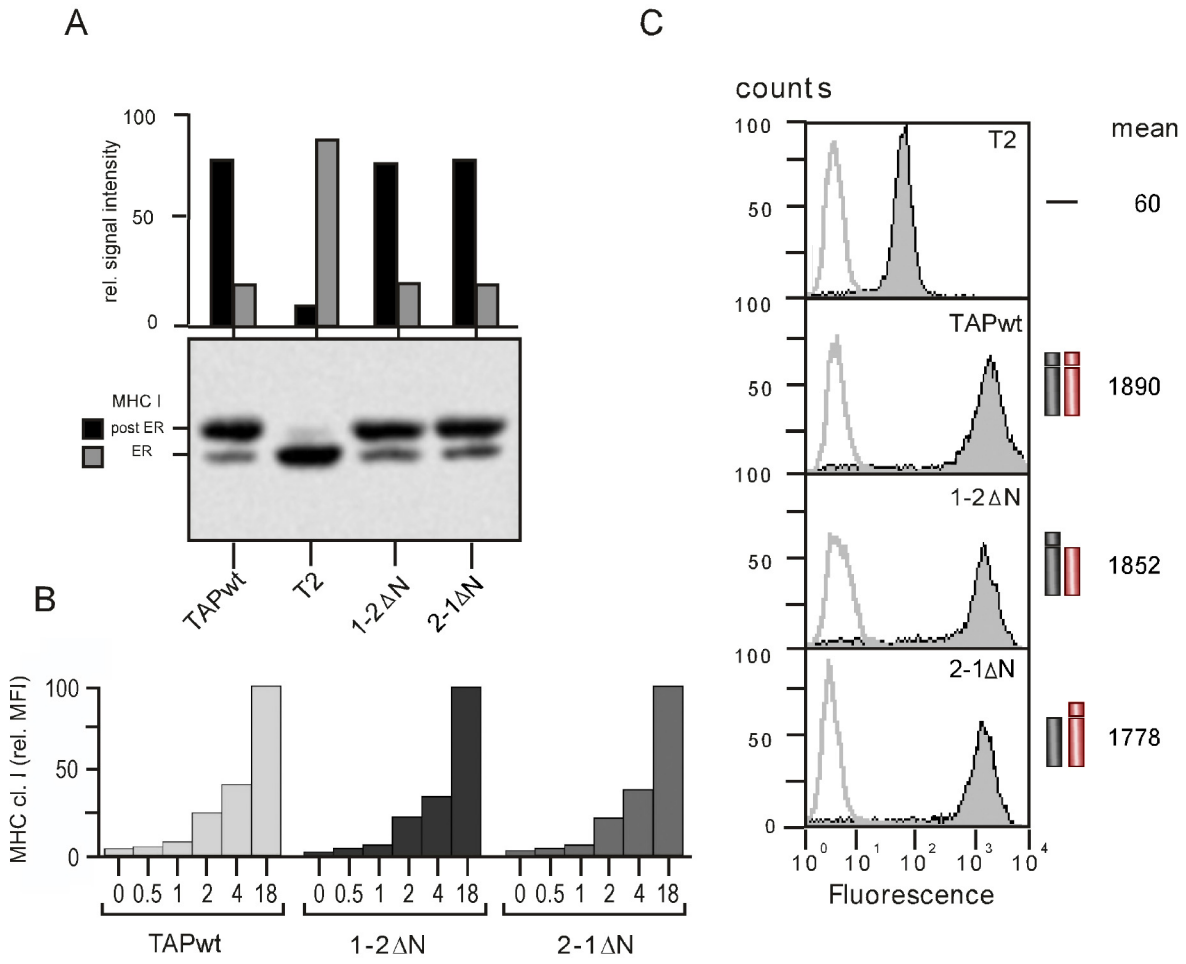

Fig. 8 **Intracellular maturation and surface expression of MHC class I in cells expressing 1-2ΔN and 2-1ΔN**
A) Intracellular maturation of MHC class I. Cell lysates were incubated with endoglycosidase H and analyzed by Western blot using the monoclonal antibody 3B10.7 (bottom). The relative intensity of the MHC class I signals quantified by densitometric scanning is shown as bar graphs (top). **B)** Surface recovery of MHC class I. Assembled MHC class I molecules were removed from the surface of T2 transfectants by a short application of acidic buffer. After neutralization of the pH cells were grown at 37°C for the indicated time before the levels of surface-HLA-B5 were determined by flow cytometry using the monoclonal antibody 4E followed by FITC-labeled secondary antibody. **C)** MHC class I surface expression. Cells were incubated with monoclonal antibody 4E followed by FITC-labeled secondary antibody and surface expression of HLA-B5 was subsequently determined by flow cytometry (filled histograms). Background staining was analyzed by incubating with secondary antibody alone (non-filled histograms).

Thus, neither the N-domain of TAP1 nor the N-domain of TAP2 is required for ER-export or surface presentation of MHC class I molecules. Furthermore, the above mentioned data provides evidence that wild-type TAP, 1-2ΔN and 2-1ΔN, despite differences in the tapasin-binding behaviour do not quantitatively differ in their transient recruitment of MHC class I for peptide-loading.

5.2 Head-to-tail-fusion of TAP chains allows stable expression of transporters lacking the N-domains in both subunits

Despite substantial efforts, a T2-transfectant stably co-expressing 1ΔN and 2ΔN could not be generated (see above). One possible explanation for this could be that a TAP transporter, which lacks both N-domains is hampered in its biogenesis. Misassembly of truncated TAP chains could lead to aggregation of the transporter and finally the accumulation of cell-toxic aggregates in the cell. Thus, to get an idea whether the complex assembly of 1ΔN with 2ΔN is impaired, an immunoprecipitation-based assay was performed, which determines the efficiency of TAP complex formation by co-depletion of TAP1 with TAP2 chains (K. Keusekotten, R. M. Leonhardt, S. Ehses and M. R. Knittler, *manuscript in preparation*). By an *in vitro*-expression system, it was recently demonstrated that stable and functional biogenesis of TAPwt requires the assembly of pre-existing TAP1 with newly synthesized TAP2, but not vice versa (K. Keusekotten, R. M. Leonhardt, S. Ehses and M. R. Knittler, *manuscript in preparation*). Therefore, either 2ΔN or TAP2 was *in vitro*-translated in the presence of purified microsomes that already contained 1ΔN or TAP1 (Fig. 9A). Following the *in vitro*-translation, the microsomes were lysed and the newly synthesized TAP2 subunits were removed in two sequential rounds of immunodepletion with the anti-TAP2 antiserum 116/5. To control for the efficiency of *in vivo*-complex assembly microsomal lysates from T2(TAPwt) cells were also subjected to immunodepletion. As shown in Fig. 9A the TAP1 chain was quantitatively removed with anti-TAP2 antibodies from solubilized membranes containing wild-type TAP, irrespective whether the TAP2 chain of the transporter had been synthesized *in vivo* (lane 6) or *in vitro* (lane 4). This suggests efficient dimerization of TAP1 and TAP2 in both cases. In contrast, the 1ΔN chain displayed a strongly diminished co-depletion with the 2ΔN chain, indicating that the assembly of

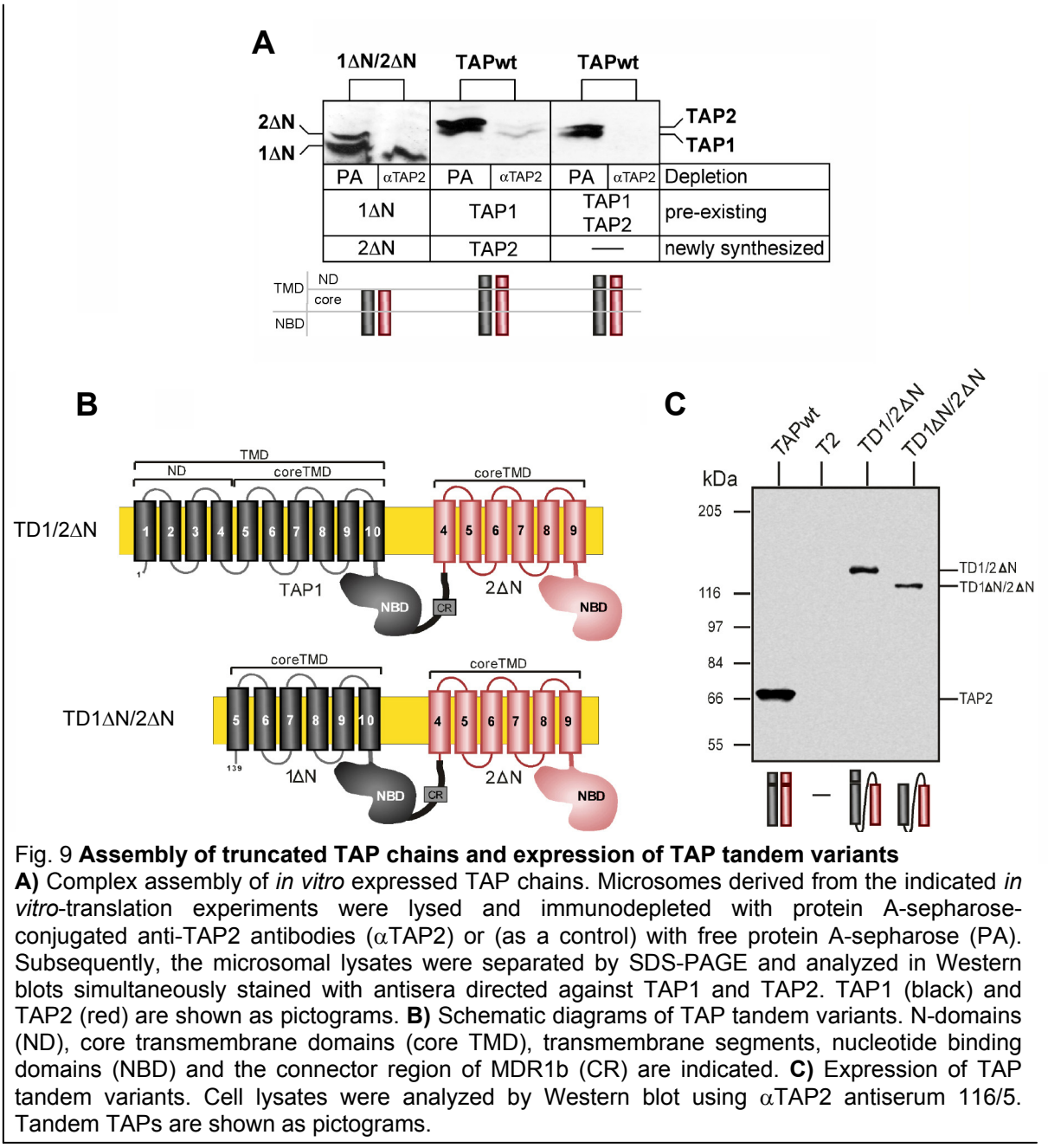

Fig. 9 **Assembly of truncated TAP chains and expression of TAP tandem variants**
A) Complex assembly of *in vitro* expressed TAP chains. Microsomes derived from the indicated *in vitro*-translation experiments were lysed and immunodepleted with protein A-sepharose-conjugated anti-TAP2 antibodies (αTAP2) or (as a control) with free protein A-sepharose (PA). Subsequently, the microsomal lysates were separated by SDS-PAGE and analyzed in Western blots simultaneously stained with antisera directed against TAP1 and TAP2. TAP1 (black) and TAP2 (red) are shown as pictograms. **B)** Schematic diagrams of TAP tandem variants. N-domains (ND), core transmembrane domains (core TMD), transmembrane segments, nucleotide binding domains (NBD) and the connector region of MDR1b (CR) are indicated. **C)** Expression of TAP tandem variants. Cell lysates were analyzed by Western blot using αTAP2 antiserum 116/5. Tandem TAPs are shown as pictograms.

the two truncated TAP chains is substantially impaired (Fig. 9A, lane 2). It cannot be excluded at present that even the observed low degree of assembly in the latter case reflects the formation of microaggregates rather than functional heterodimers. Nevertheless, the *in vivo*-data (Fig. 7A) shows that neither 1ΔN nor 2ΔN has a general defect in complex assembly. Thus, it was speculated that a head-to-tail fusion of both truncated TAP chains to a single gene might circumvent the observed expression problems and allow for the generation of a TAP variant lacking both N-domains in T2 cells. The order of TMDs and NBDs within such a gene would

resemble the genetic structure of so-called tandem ABC transporters, which encode the four characteristic domains of this transporter family (two TMDs and two NBDs) on one chain. In particular, a 1ΔN-2ΔN fusion protein would resemble the TAP-related tandem multidrug resistance transporter 1 (MDR1) (Gros et al, 1986) as this transporter contains only the conserved six α-helices of the core TMD of ABC transporters, whereas any homologous sequence corresponding to the N-domains of TAP is lacking (Seigneuret et al, 2003). However, in MDR1 (as generally in tandem transporters) a flexible connector region of approx. 60 amino acids joins the two homologous halves of the protein (Hrycyna et al, 1998). The presence of this connector region was shown to be essential for transport function of MDR1 as its deletion results in an ATP-hydrolysis-deficient and transport-inactive protein (Hrycyna et al, 1998).

Thus, a TAP-tandem-construct was generated, in which the cDNAs of 1ΔN and 2ΔN were linked by the connector region of murine MDR1b (Fig. 9B, lower panel; see section 4.2) (Gros et al, 1986). In addition, a second TAP-fusion construct was made containing wild-type TAP1 instead of 1ΔN (Fig. 9B upper panel; see section 4.2). The resulting tandem constructs (named TD1ΔN/2ΔN and TD1/2ΔN) were transfected into T2 cells and analyzed for steady-state expression by Western blot. Fig. 9C shows that both, TD1ΔN/2ΔN and TD1/2ΔN were stably expressed in the transfectants. Next, the subcellular distribution of the two tandem TAP variants was analyzed by immunofluorescence. Fig. 10A shows that comparable to TAPwt both transporters co-localized with KDEL-containing polypeptides in the endoplasmic reticulum. For functional characterisation of TD1/2ΔN or TD1ΔN/2ΔN peptide-binding (Fig. 10B) and translocation (Fig. 10C) were analyzed by the standard methods described above. Both tandem variants displayed detectable peptide-cross-linking suggesting that

TD1/2ΔN and TD1ΔN/2ΔN contain functional substrate binding sites (Fig. 10B). Moreover, the transport activities obtained for TD1/2ΔN and TD1ΔN/2ΔN (Fig. 10C) were roughly within the range of the wild-type transporter when normalized for the expression levels of the different TAP variants. Thus, TAP lacking both N-domains is functional when stably expressed in T2 cells as a head-to-tail-fusion protein.

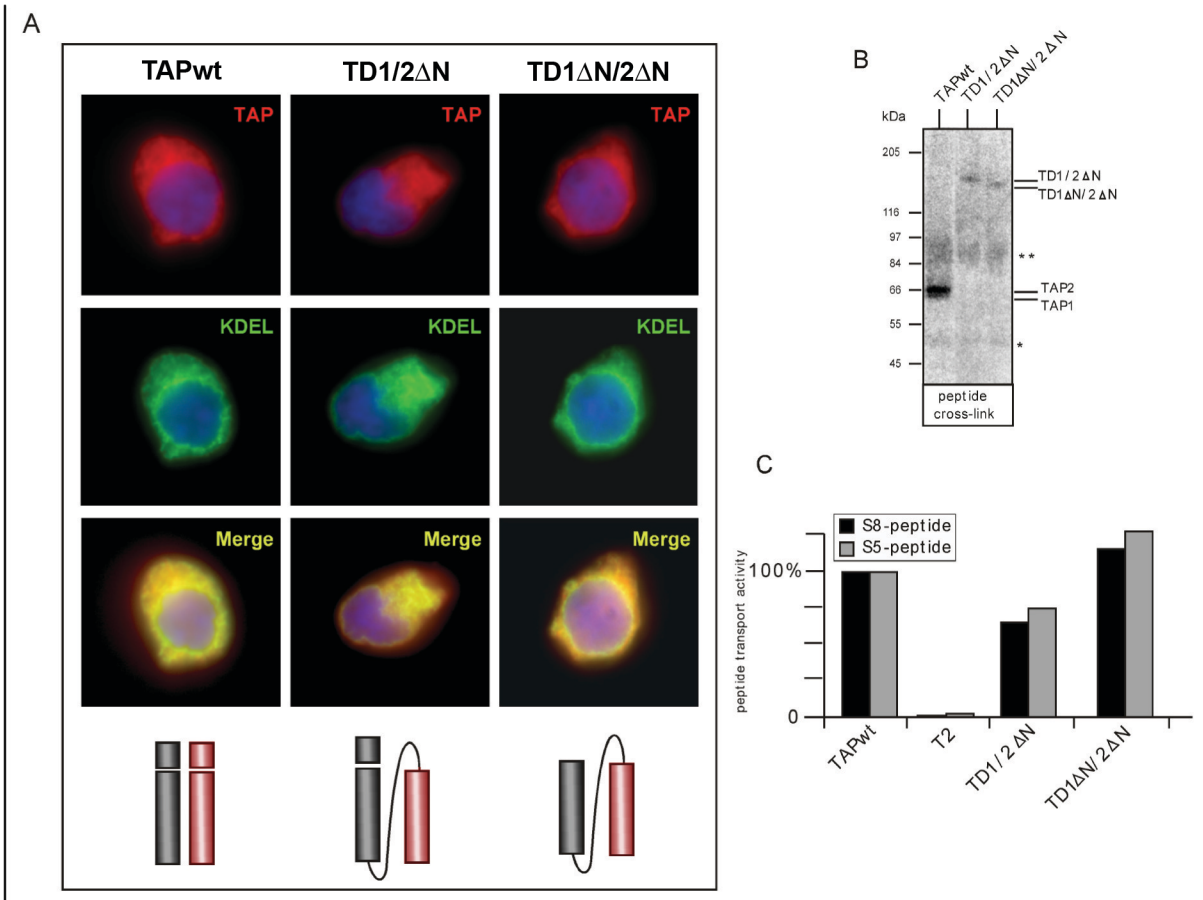

Fig. 10 **Subcellular localization and transport function of tandem TAPs**
(A) Intracellular localization of tandem TAP variants. T2 cells, expressing the indicated wild-type or tandem TAP variant were fixed, permeabilized and co-stained with antibodies directed against the ER retention signal KDEL (MAC 256) and TAP2 (116/5), followed by Alexa 488- and Alexa 546-conjugated secondary antibodies. Cells were subsequently analyzed using a Zeiss Axioplan II fluorescence microscope. **(B)** Peptide-binding of TAPwt, TD1/2ΔN and TD1ΔN/2ΔN. Permeabilized cells were incubated with radiolabeled and HSAB-conjugated peptide S5 (TVDNKTRYV). After UV-cross-linking and membrane lysis, TAP was immunoisolated with αTAP2 antiserum. Asterisks indicate unidentified cross-link products. **(C)** Peptide transport activity. Cells were permeabilized and incubated with radiolabeled peptides S5 (TVDNKTRYV) or S8 (TVDNKTRYR) in the presence of ATP. The transport activity measured for TD1/2ΔN and TD1ΔN/2ΔN is expressed as percentage of the transport activity (quantified by γ-counting) obtained for TAPwt after normalization for TAP expression.

To analyze the complex formation of the tandem transporters with tapasin and MHC class I, immunoprecipitation studies on TD1/2ΔN and TD1ΔN/2ΔN were performed. TAP and MHC class I molecules were immunoisolated from digitonin lysates with

specific antibodies and the co-isolated polypeptides were analyzed by Western blot (Fig. 11A). In line with the findings on TAP variant 1-2ΔN (Fig. 7A and Fig. 16), the related tandem transporter TD1/2ΔN retained the capacity to interact with tapasin and MHC class I whereas TD1ΔN/2ΔN, which lacks both N-terminal tapasin binding

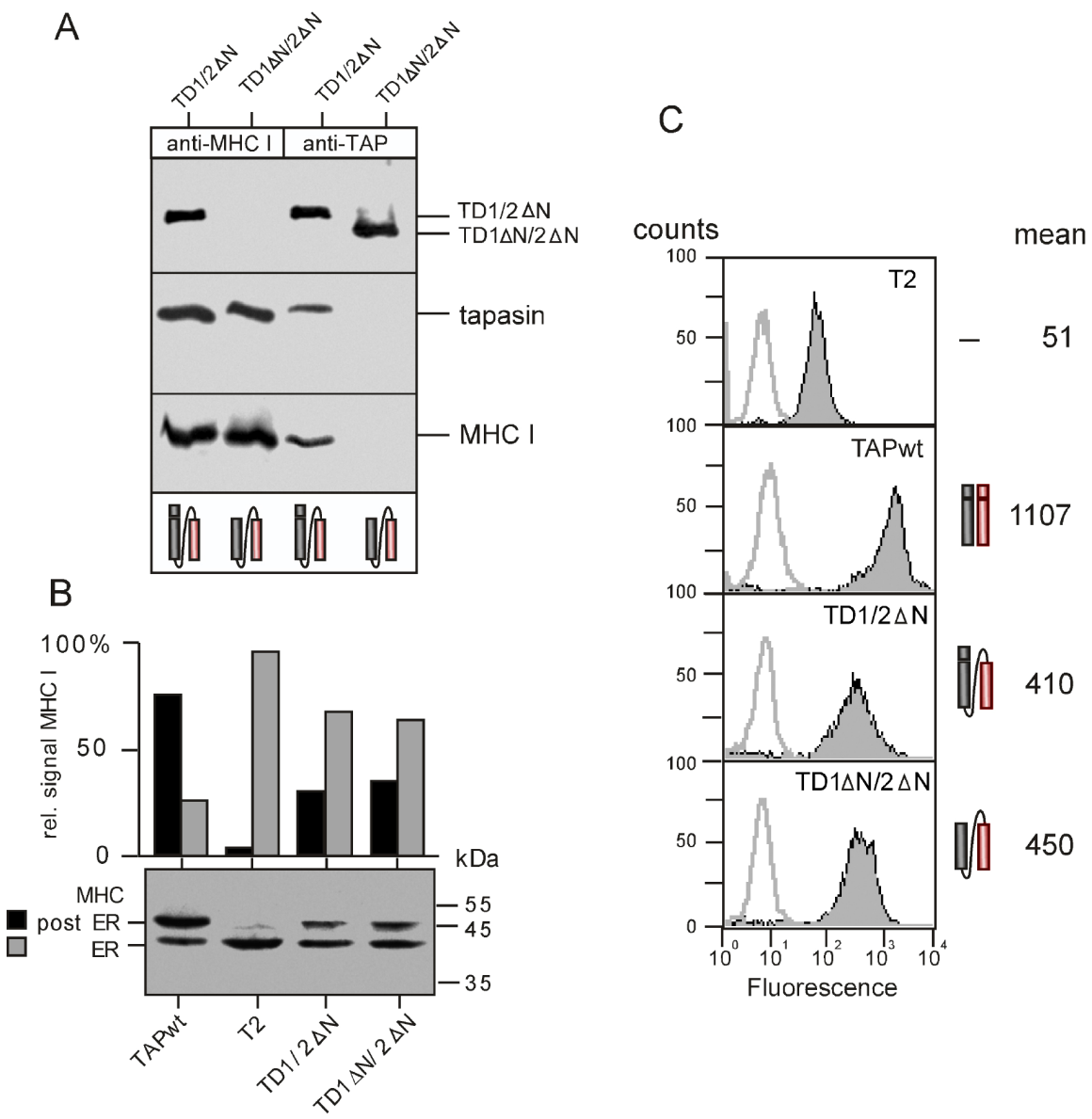

Fig. 11 **PLC formation and functional characterization of tandem TAPs**
(A) TAP/tapasin-interaction. Digitonin lysates of transfectants expressing tandem TAP variants were subjected to immunoprecipitation using the αTAP2 antiserum 116/5 or the monoclonal αMHC class I antibody 3B10.7. Precipitates were analyzed in Western blots probed for TAP2, tapasin or MHC class I heavy chains. Tandem TAPs are shown as pictograms. **(B)** Intracellular maturation of MHC class I. Cell lysates were incubated with endoglycosidase H and analyzed by Western blot using the monoclonal antibody 3B10.7 (bottom). The relative intensity of the MHC class I signals quantified by densitometric scanning is shown as bar graphs (top). **C)** MHC class I surface expression. Cells were incubated with monoclonal antibody 4E followed by FITC-labeled secondary antibody and surface expression of HLA-B5 was subsequently determined by flow cytometry (filled histograms). Background staining was analyzed by incubating with secondary antibody alone (non-filled histograms).

sites, is completely excluded from the PLC (Fig. 11A). Next, the capability of TD1/2ΔN and TD1ΔN/2ΔN to deliver peptide substrates for the loading onto MHC class I molecules was investigated. Since the expression levels of the functional tandem transporters were lower than the expression levels of wild-type TAP (Fig. 9C), the cell lines T2(TD1/2ΔN) and T2(TD1ΔN/2ΔN) displayed only a partial restoration of intracellular processing (Fig. 11B) and surface expression (Fig. 11C) of MHC class I. However, it is interesting to note that despite the differences in the tapasin/MHC class I recruitment by the two tandem transporters, the cell lines expressing TD1/2ΔN and TD1ΔN/2ΔN showed very similar steady-state distributions of ER and post-ER MHC class I (Fig. 11B) and comparable amounts of surface HLA-B5 (Fig. 11C). This implies that under conditions, in which physical interaction between TAP and tapasin is impaired, newly synthesized MHC class I molecules can take alternative routes of peptide-loading outside the TAP-associated PLC as long as suitable peptides are delivered by a functional transporter.

5.3 Assembled and non-assembled TAP1 chains use different TMD-subregions for tapasin binding

Comparison of the experiments on the tandem transporters and the heterodimeric variants suggests that both N-domains of TAP serve as functional interaction sites for tapasin and MHC class I molecules. However, the two TAP subunits are known to bind the components of the PLC independently from each other (Antoniou et al, 2002b; Daumke et al, 2001; Newitt et al., 1999; Raghuraman et al, 2002). Thus, the question arises whether the interaction between tapasin and the N-domains depends on the heterodimerization of TAP1 and TAP2 or reflects an intrinsic property of the two TAP subunits. To address this question, 1ΔN and 2ΔN were stably transfected into T2 cells as single TAP chains. Analysis of the steady-state expression levels by

Western blot revealed that both variants were expressed at levels comparable to that of the corresponding wild-type TAP subunits (Fig. 12A). Both chains retained the normal nucleotide binding behaviour of the wild-type TAP subunits (Alberts et al, 2001; Ehses et al, 2005) as the TAP1-variant bound both ATP and ADP whereas the TAP2-variant associated solely with ADP (Fig. 12B).

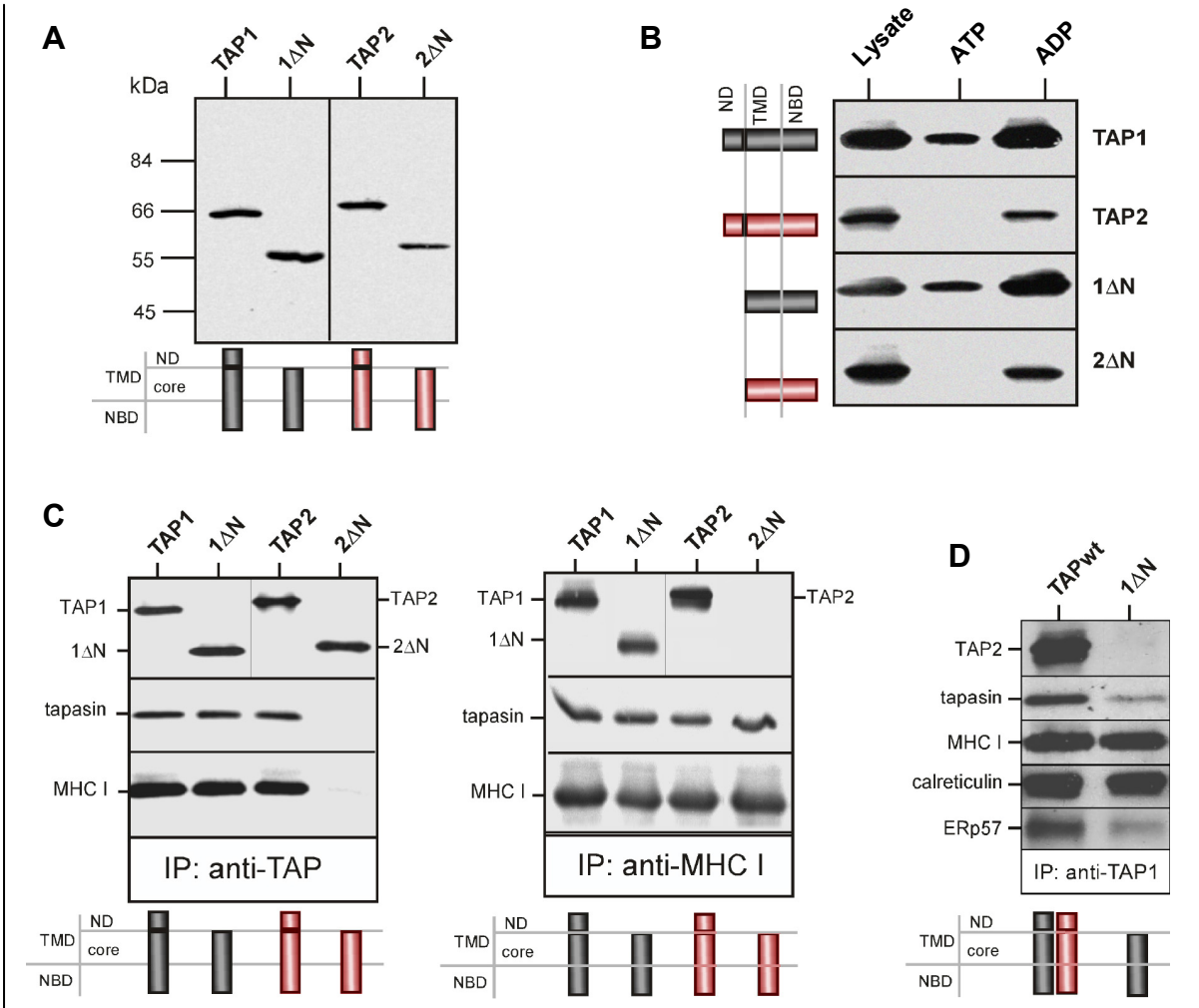

Fig. 12 **Expression, nucleotide binding and PLC formation of N-terminally truncated TAP subunits**
(A) Expression of N-terminally truncated TAP chains. Cell lysates were analyzed in Western blots probed for TAP1 or TAP2. TAP1 (black) and TAP2 (red) are shown as pictograms. **(B)** Nucleotide binding properties of single expressed TAP subunits. Membrane fractions of T2 transfections were lysed before incubation with different nucleotide-agaroses. Bound proteins were eluted with SDS-containing sample buffer and analyzed in Western blots probed for TAP1 and TAP2. **(C)** TAP/tapasin-interaction. Digitonin lysates of transfectants expressing N-terminally truncated TAP subunits were subjected to immunoprecipitation using the αTAP1 antiserum D90 (left panel, left half), αTAP2 antiserum 116/5 (left panel, right half) or the monoclonal αMHC class I antibody 3B10.7 (right panel). Precipitates were analyzed in Western blots probed for TAP1, TAP2, tapasin or MHC class I heavy chains. TAP subunits are shown as pictograms. **(D)** Association of 1ΔN or wild-type TAP with accessory chaperones of the PLC. Digitonin lysates of transfectants expressing wild-type TAP or the 1ΔN single chain were subjected to immunoprecipitation using the αTAP1 antiserum D90. Precipitates were analyzed in Western blots probed for TAP2, tapasin, MHC class I heavy chains, Calreticulin or ERp57.

To investigate whether the single TAP chain variants become recruited into the PLC, 1ΔN and 2ΔN were immunoprecipitated from lysates of the cell lines T2(1ΔN) or T2(2ΔN) with either anti-TAP1 (D90) or anti-TAP2 (116/5) antisera (Fig. 12C). The co-isolated polypeptides were analyzed in Western blots using specific antibodies recognizing tapasin and MHC class I. Consistent with the data on the tandem transporter variant TD1ΔN/2ΔN (Fig. 11A) the single chain 2ΔN was not found in association with any of the components of the PLC at all (Fig. 12C left panel and data not shown). Surprisingly however, the single expressed 1ΔN fully retained the ability to interact with tapasin and MHC class I suggesting that the core TMD of the TAP1 chain allows efficient complex formation (Fig. 12C left panel). To verify this, immunoprecipitations using anti-MHC class I antibodies were performed (Fig. 12C, right panel). In accordance with the results of the anti-TAP immunoprecipitations, 1ΔN, but not 2ΔN co-precipitated with MHC class I molecules (Fig. 12C, right panel). Furthermore, to compare PLCs formed via the N-domains of wild-type TAP with PLCs formed via the core TMD of TAP1, lysates of the cell lines T2(TAPwt) and T2 (1ΔN) were subjected to immunoprecipitation using the TAP1-specific antiserum D90. Co-isolated polypeptides were subsequently separated by SDS-PAGE and analyzed by Western blot (Fig. 12D). Consistent with the finding that wild-type TAP does not associate with more MHC class I than the two variants 1-2ΔN or 2-1ΔN (Fig. 7B and Fig. 16) the amounts of MHC class I co-isolated with 1ΔN and the wild-type transporter were roughly identical (Fig. 12D). Additionally, the amounts of calreticulin co-precipitating with 1ΔN and TAPwt were also very similar. Thus it seems that the fully assembled transporter does not even associate with significantly higher levels of MHC class I and calreticulin as the 1ΔN single chain. However, consistent with the finding that TAPwt interacts with tapasin via both TAP1 and TAP2 (Fig. 16) whereas

1ΔN binds tapasin solely through its core TMD the levels of co-precipitated tapasin were significantly higher in the case of the wild-type transporter than of the single chain 1ΔN (Fig. 12D). Consistent with the model that tapasin contributes to the recruitment of ERp57 into the PLC (Dick et al, 2002; Diedrich et al, 2001; Harris et al, 2001) the levels of TAPwt-bound ERp57 versus 1ΔN-bound ERp57 were also markedly higher (Fig. 12D).

Furthermore, the subcellular distribution of the truncated TAP chains 1ΔN and 2ΔN was analyzed by indirect immunofluorescence. Fig. 13 shows that both subunits properly co-localized with KDEL-containing proteins in the ER. Thus, neither N-domain is essential for ER-targeting of the respective TAP chain. Moreover, this

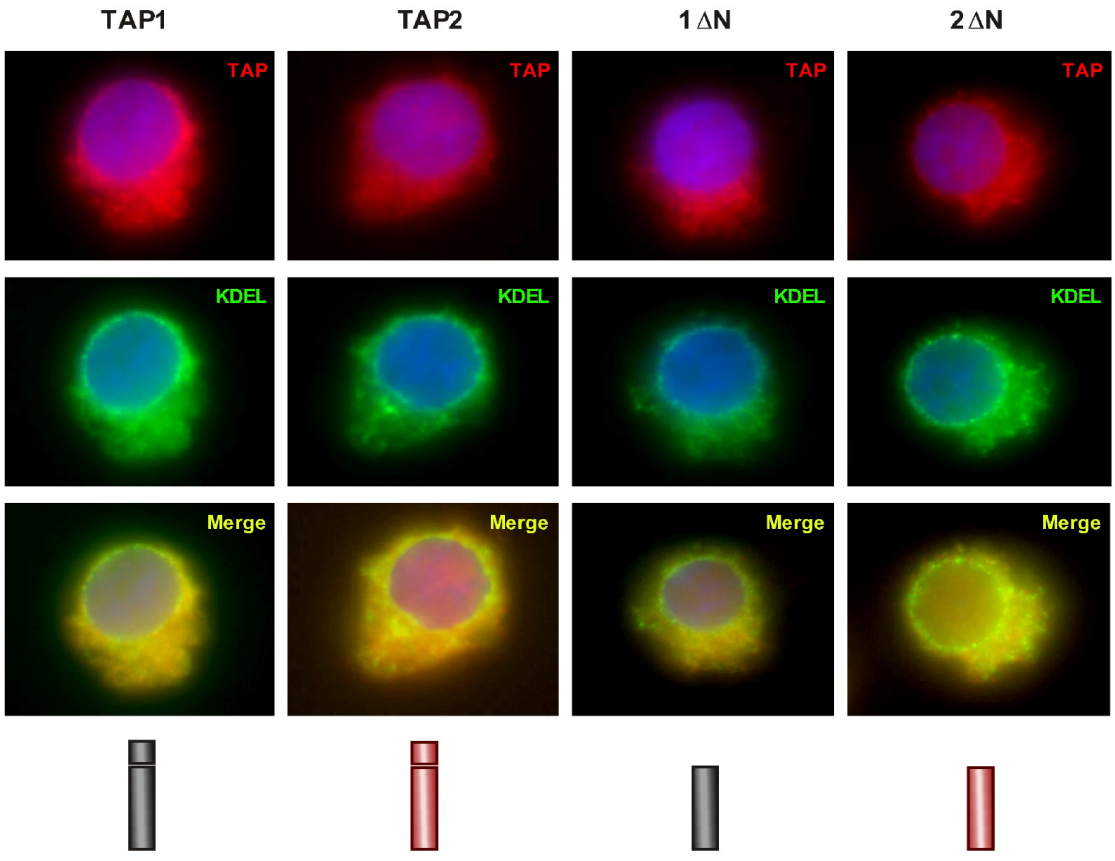

Fig. 13 **Subcellular localization of TAP single chain variants**
Intracellular localization of mutant TAP chains. T2 cells, expressing the indicated wild-type or mutant TAP subunit were fixed, permeabilized and co-stained with antibodies directed against the ER retention signal KDEL (MAC 256) and either TAP1 (D90) or TAP2 (116/5), followed by Alexa 488- and Alexa 546-conjugated secondary antibodies. Cells were subsequently analyzed using a Zeiss Axioplan II fluorescence microscope.

experiment excludes that the different tapasin-interaction of 1ΔN and 2ΔN is caused by a distinctive intracellular localization of the truncated subunits.

Taken together, it seems that depending on the assembly state of TAP1 different subregions of TMD1 are used for the interaction with tapasin whereas in TAP2 the tapasin-interaction is exclusively mediated by the N-domain.

5.4 TAP variants 1-2ΔN and 2-1ΔN show different effects on the quality control of antigen presentation

Tan and co-workers recently reported that MHC class I-loading with peptides conferring high complex stability requires the tapasin-mediated introduction of TAP into the PLC (Tan et al, 2002). To investigate whether and to what extend the tapasin-docking sites are required in the quality control of MHC class I processing the correlation between the thermostability of MHC class I molecules and the affinity for their peptide cargo was applied. The ability of MHC class I molecules to withstand thermal denaturation in cell extracts is directly related to the binding affinity of their peptide ligands (Williams et al, 2002). Consequently, at temperatures ≥37°C only the population of stably loaded MHC class I molecules resists irreversible disassembly (Baas et al, 1992; Williams et al, 2002). To explore the thermostability of assembled MHC class I in the established cell lines, lysates of the different transfectants were prepared and subsequently incubated for 1 hour at 4 °C or 37 °C (Fig. 14A and Fig. 14B). The integrity of MHC class I complexes was tested by immunoprecipitations with the HLA-B5-specific β_2m-dependent monoclonal antibody 4E (Trapani et al, 1989); Tector et al, 1995). In transfectants expressing wild-type TAP or 2-1ΔN the majority of HLA-B5 was found to be heat-stable (Fig. 14A lanes 2 and 5, for quantification see Fig. 14B) whereas in cells expressing 1-2ΔN or no TAP most of the MHC class I-peptide complexes did not survive the incubation at 37°C (Fig. 14A

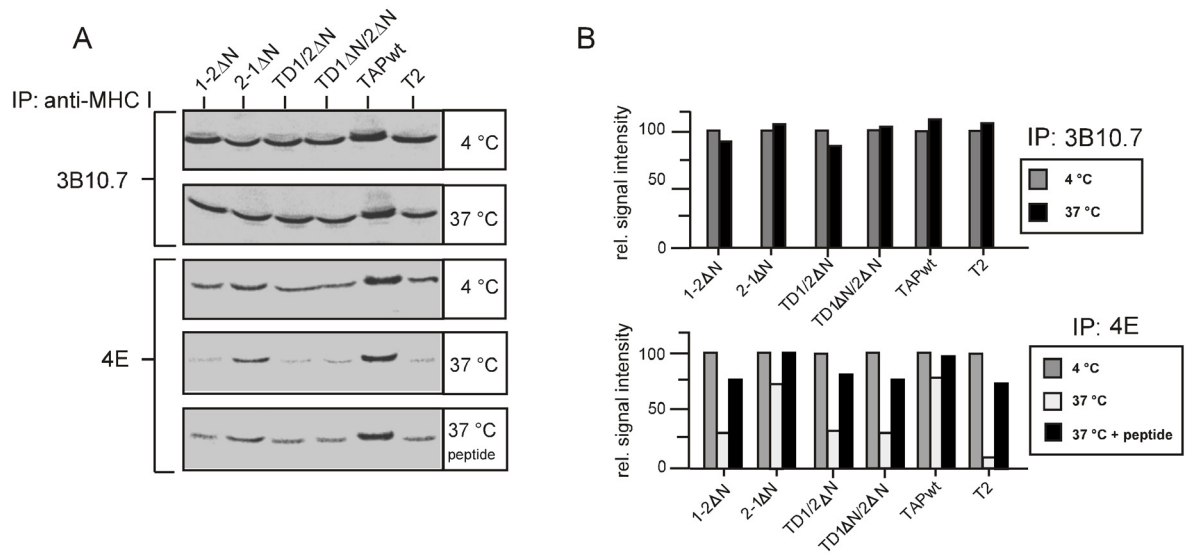

Fig. 14 ***In vitro*** **thermostability of MHC class I in detergent extracts**
(A) Thermostability of MHC class I in cell extracts. Lysates of T2 transfectants were pre-treated or not with 10 μM HLA-B5 binding peptide EBV-3C/881-9 (QPRAPIRPI) and incubated for 1h at 4 °C or 37 °C. The thermostability of HLA-B5 was analyzed by immunoprecipitation of MHC class I from these extracts using either the conformation-independent monoclonal antibody 3B10.7 (top) or the β_2-microglobulin-dependent monoclonal antibody 4E (bottom). Precipitates were analyzed in Western blots probed with the antibody 3B10.7. **(B)** The levels of precipitated MHC class I heavy chain were quantitated from the Western blot shown in Fig. 14A by densitometric scanning and peak integrals were plotted as bar graphs in arbitrary units.

lanes 1 and 6, for quantification see Fig. 14B). Instability of HLA-B5 could be also seen for transfectants expressing tandem transporters TD1/2ΔN or TD1ΔN/2ΔN (Fig. 14A lanes 3 and 4, for quantification see Fig. 14B). In contrast, no temperature effects were observed in control experiments where immunoisolations were performed with the conformation-independent anti-MHC class I antibody 3B10.7 (Fig. 14A, for quantification see Fig. 14B). To elucidate whether the observed heat-sensitivity of the MHC class I molecules is due to suboptimal peptide-binding, lysates were pre-incubated with the high affinity HLA-B5 peptide ligand EBV-3C/881-9 (QPRAPIRPI, in single letter amino acid code) (Hill et al, 1995) before incubation at 37°C. As can be seen from Fig. 14A (for quantification see Fig. 14B), the pre-treatment with peptides lead to a significant stabilization of HLA-B5 in cells expressing 1-2ΔN, TD1/2ΔN or TD1ΔN/2ΔN or no TAP at all. Thus, the presence of

the N-domain in TAP2, albeit not being essential for MHC class I processing, appears to be required for the formation of stable MHC class I complexes. To validate this idea, the thermostability of intracellular and cell surface HLA-B5 complexes in intact cells was analyzed. For these studies the transfectants T2(TAPwt), T2 (1-2ΔN) and T2 (2-1ΔN) were used as these display comparable levels of TAP expression (Fig. 5B). Since assembled MHC class I molecules have a much higher heat-resistance under physiological conditions than in detergent extracts (Batalia et al, 2000), transfectants were heat-shocked for 10 min at 40, 50 and 60°C or not heat-shocked at all. To determine thermostability of surface HLA-B5, cells were directly immunostained with the monoclonal antibody 4E whereas for the analysis of intracellular HLA-B5, transfectants were first permeabilized and then immunostained for flow cytometry. The experiments in Fig. 15A show that the cell lines expressing TAPwt or variant 2-1ΔN were characterized by a high thermostability of intracellular HLA-B5 whereas in non-transfected T2 cells only 20% of intracellular HLA-B5 remained stable at 40°C and ≤10% at temperatures above 50°C. In line with the experiments shown in Fig. 14, the T2-transfectant expressing variant 1-2ΔN showed a remarkable thermolability of intracellular HLA-B5 at temperatures >40°C (40 and 70% reduction of the intracellular immunostaining at 50 and 60°C, respectively). However, most surprisingly, all analyzed transfectants displayed a comparable high thermostability of surface HLA-B5. Accordingly, in the presence of the drug brefeldin A (BFA), which blocks the ER-Golgi transport, the time course of MHC class I-decay from the cell surface was determined to be almost identical ($t_{1/2}$ = 15h) among the cell lines T2(1-2ΔN), T2(2-1ΔN) and T2(TAPwt) (Fig. 15B), also indicating similar surface class I stability in these transfectants. Taken together, this suggests that in the cell line T2(1-2ΔN) the population of HLA-B5 molecules on the cell surface is physically more stable than the intracellular fraction of HLA-B5.

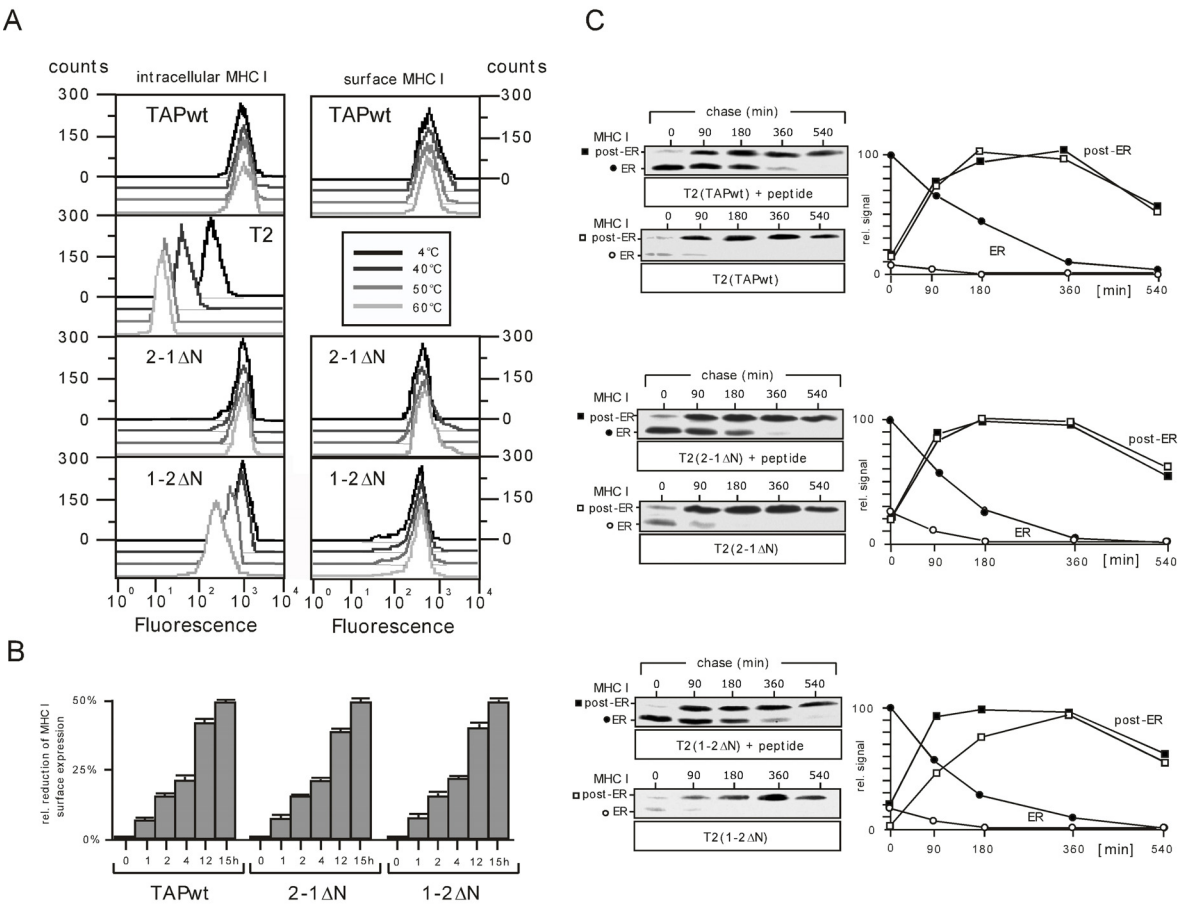

Fig. 15 **Stability of MHC class I molecules in intact cells**
(A) Thermostability of intracellular and cell surface MHC class I. Transfectants were collected in PBS / 0,1% NaN_3 before incubation at 4, 40, 50 or 60 °C for 10 min. To examine the thermostability of surface HLA-B5, cells were directly immunostained with the monoclonal αMHC class I antibody 4E. To analyze the thermostability of intracellular HLA-B5, transfectants were fixated, permeabilized and stained with antibody 4E. After incubation with FITC-labeled secondary antibody HLA-B5 levels were determined by flow cytometry. **(B)** Survival of MHC class I molecules on the cell surface. Transfectants were treated with brefeldin A (BFA) that inhibits protein export from the ER. After culturing the cells for the time indicated, MHC class I surface molecules were stained with antibody 4E. Results of the flow cytometry are presented as percentage of the reduction in the mean fluorescence intensities (MFI) at 1, 2, 4, 12 and 15h compared with the MFI at 0h. **(C)** Maturation and thermostability of MHC class I. Transfectants were pulse-labeled for 30 min and chased for the indicated times. Subsequently, cells were lysed in the presence or absence of 10 μM peptide EBV-3C/881-9 (QPRAPIRPI) and incubated for 1h at 37 °C. Immunoprecipitates recognized by the antibody 4E were digested with endoglycosidase H and separated by SDS-PAGE (left panel). Obtained signals of MHC class I heavy chains (ER and post-ER) were quantitated and the bands with the highest intensity were set to 100% (right panel).

To investigate whether stabilization of HLA-B5 complexes can occur during the intracellular transport to the cell surface, pulse-chase experiments were performed with the different transfectants (Fig. 15C). Cell lysates at different time points were heat-treated at 37°C for 1h in the presence or absence of the MHC class I-stabilizing peptide EBV-3C/881-9 (QPRAPIRPI, in single letter amino acid code) (Hill et al, 1995) and subsequently incubated with endoglycosidase H. The fraction of

thermostable MHC class I molecules was assessed by immunoprecipitation with the conformation-sensitive monoclonal antibody 4E. Consistent with the experiments shown in Fig. 8A, Fig. 8B and Fig. 8C the ER-export rate of total HLA-B5 was found to be similar in T2(TAPwt), T2(1-2ΔN) and T2(2-1ΔN) as determined in the presence of stabilizing peptide (Fig. 15C, closed symbols). Furthermore, without the addition of external peptides MHC class I molecules of T2(TAPwt) or T2(2-1ΔN) showed high thermostability when they had left the ER (Fig. 15C, open symbols). However, the situation was different in the cell line T2(1-2ΔN) as here MHC class I molecules were thermolabile even after their export from the ER (Fig. 15C, open symbols). Moreover, comparison of the signals of the Endo H-resistant MHC class I isolated in the presence or absence of stabilizing peptides revealed that in T2(1-2ΔN) the ER-exported MHC class I molecules improved their thermostability during the process of post-ER maturation (Fig. 15C).

The experiments described above suggest that PLCs formed by the TAP variant 1-2ΔN have lost their quality control function in the generation of stably loaded MHC class I. Furthermore, the presented data provides evidence that structural stability of MHC class I complexes can be rescued in the late compartments of the secretory route.

5.5 The N-domain of TAP2 is essential for the structural integrity of the PLC

To elucidate the molecular basis for the defective quality control of MHC class I processing in transfectants expressing 1-2ΔN the composition of PLCs comprising the TAP variants TAPwt, 1-2ΔN or 2-1ΔN was analyzed. The proper assembly of the PLC may be of central significance as in addition to tapasin, also the accessory chaperones ERp57 and calreticulin are thought to play a critical role in the quality

control of MHC class I loading (Wright et al, 2004). Recent models propose that the functional interplay of all three components is required for the optimization of the peptide cargo for MHC class I within the PLC (Gao et al, 2002; Dick et al, 2002). This is also reflected in the high cooperativity of interactions among the different PLC components (Harris et al, 2001; Hughes et al, 1998; Momburg et al, 2002; Sadasivan et al, 1996). Therefore, the complex formation of 1-2ΔN and 2-1ΔN with ERp57 and calreticulin was examined by immunoprecipitation, using the TAP2-specific antiserum 116/5. Additionally, the association of the transporter variants with calnexin, which is thought to be a component of the so-called precursor-complex that precedes the formation of the PLC (Diedrich et al, 2001) was analyzed. The TAP immunoblots of SDS-PAGE-separated precipitates showed comparable amounts of wild-type and truncated TAP chains, demonstrating that immunoisolations from all three cell lines had been equally efficient (Fig. 16). In accordance with the experiments shown in

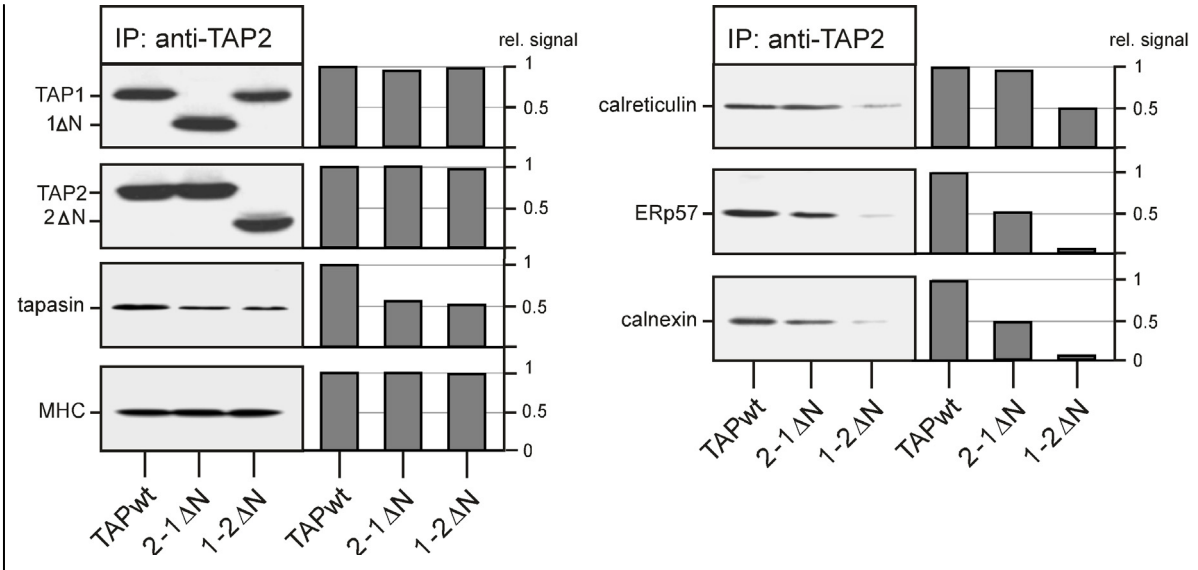

Fig. 16 **Composition of PLCs comprising TAP variants 2-1ΔN and 1-2ΔN**
Comparative analysis of PLC formation by wild-type TAP, 2-1ΔN and 1-2ΔN. Immunoblots of SDS-PAGE-separated proteins from αTAP2 precipitates were probed for TAP1, TAP2, tapasin, MHC class I heavy chains, ERp57, calreticulin or calnexin. The relative intensities of signals quantified by densitometric scanning are shown as bar graphs.

Fig. 7, comparable co-isolation of MHC class I was also observed (Fig. 16). Furthermore, in support of the model that calreticulin becomes inserted into the PLC together with MHC class I (Diedrich et al, 2001) identical amounts of this chaperone

were found in the complexes of wild-type TAP and 2-1ΔN (Fig. 16). It is worth to note that this is reminiscent of the similar levels of MHC class I and calreticulin co-precipitating with TAPwt and the single chain 1ΔN (Fig. 12D). However, the amount of tapasin, ERp57 and calnexin co-isolated with 2-1ΔN equalled half the amount co-isolated with the wild-type transporter indicating that in 2-1ΔN solely the N-domain of TAP2 is involved in the formation of precursor and/or peptide-loading complexes (Fig. 16). As seen before (Fig. 7), the interaction of 1-2ΔN with tapasin and MHC class I resembled the properties of complexes formed by variant 2-1ΔN (Fig. 16). Yet, in striking contrast to 2-1ΔN, the quantities of accessory chaperones co-precipitated with 1-2ΔN were significantly diminished. The amounts of co-isolated ERp57, calnexin and calreticulin corresponded to 10, 15 and 40% of that found in the complex of TAPwt. This indicates that either the steady state levels of these chaperones in PLCs comprising 1-2ΔN are drastically reduced or that the interactions among the polypeptides within these PLCs are substantially weaker so that the accessory components are lost during the immunoprecipitation procedure. In any case, despite efficient recruitment of tapasin and MHC class I to the N-domain of TAP1 in 1-2ΔN, the formation of precursor- and/or peptide-loading- complexes seems to be markedly disturbed in the cooperative assembly of accessory chaperones. It is reasonable to assume that this is directly responsible for the defective quality control of MHC class I loading observed in cells expressing variant 1-2ΔN.

5.6 Calnexin is part of the peptide loading complex in T2 cells expressing TAP

The experiments depicted in Fig. 16 showed that the instability of intracellular MHC class I molecules in the cell line 1-2ΔN correlated with diminished co-isolation of

calnexin with TAP. This was surprising, as calnexin had previously not been reported to be a component of the PLC in human cells, but instead to transiently associate via tapasin with TAP in a so-called precursor complex before MHC class I becomes inserted along with calreticulin (Diedrich et al, 2001). Nevertheless, this view on the assembly of the PLC contrasts with observations regarding the mature loading complex in murine cells that was shown to comprise significant levels of the membrane-anchored lectin chaperone (Suh et al, 1996). Thus, to address the question whether calnexin associates with the PLC in human T2 cells expressing TAPwt, 1-2ΔN or 2-1ΔN, respective cell extracts were subjected to consecutive immunoprecipitation to isolate mature loading complexes, which contain TAP and MHC class I at the same time. Therefore, in a first step TAP-associated polypeptides were precipitated from digitonin lysates of the cell lines T2(TAPwt), T2(1-2ΔN) and T2(2-1ΔN) using the TAP2-specific antiserum 116/5. Subsequently, bound protein complexes were eluted by competition with a peptide corresponding to the C-terminus of TAP2 (EQDVYAHLVQQRLEA, in single letter amino acid code) (Momburg et al, 1992). An aliquot of this eluate was saved for Western blot analysis of TAP-associated proteins (Fig. 17A, lanes 1, 2 and 3) before complexes were re-precipitated with the monoclonal anti-MHC class I antibody 3B10.7. Isolated PLCs were separated by SDS-PAGE and analyzed by Western blot using antisera directed against calnexin, TAP1 or MHC class I (Fig. 17A, lanes 4, 5 and 6). Fig. 17A (lane 4) shows that significant amounts of calnexin were found in complexes that simultaneously comprised TAPwt and MHC class I, indicating that the lectin chaperone is indeed a component of the mature PLC in T2 cells expressing the wild-type transporter. However, the total amount of calnexin associated with TAP was determined to be somewhat larger than its amount in TAP-MHC class I complexes (Fig. 17A, compare lane 1 and 4). This difference may reflect the co-existence of

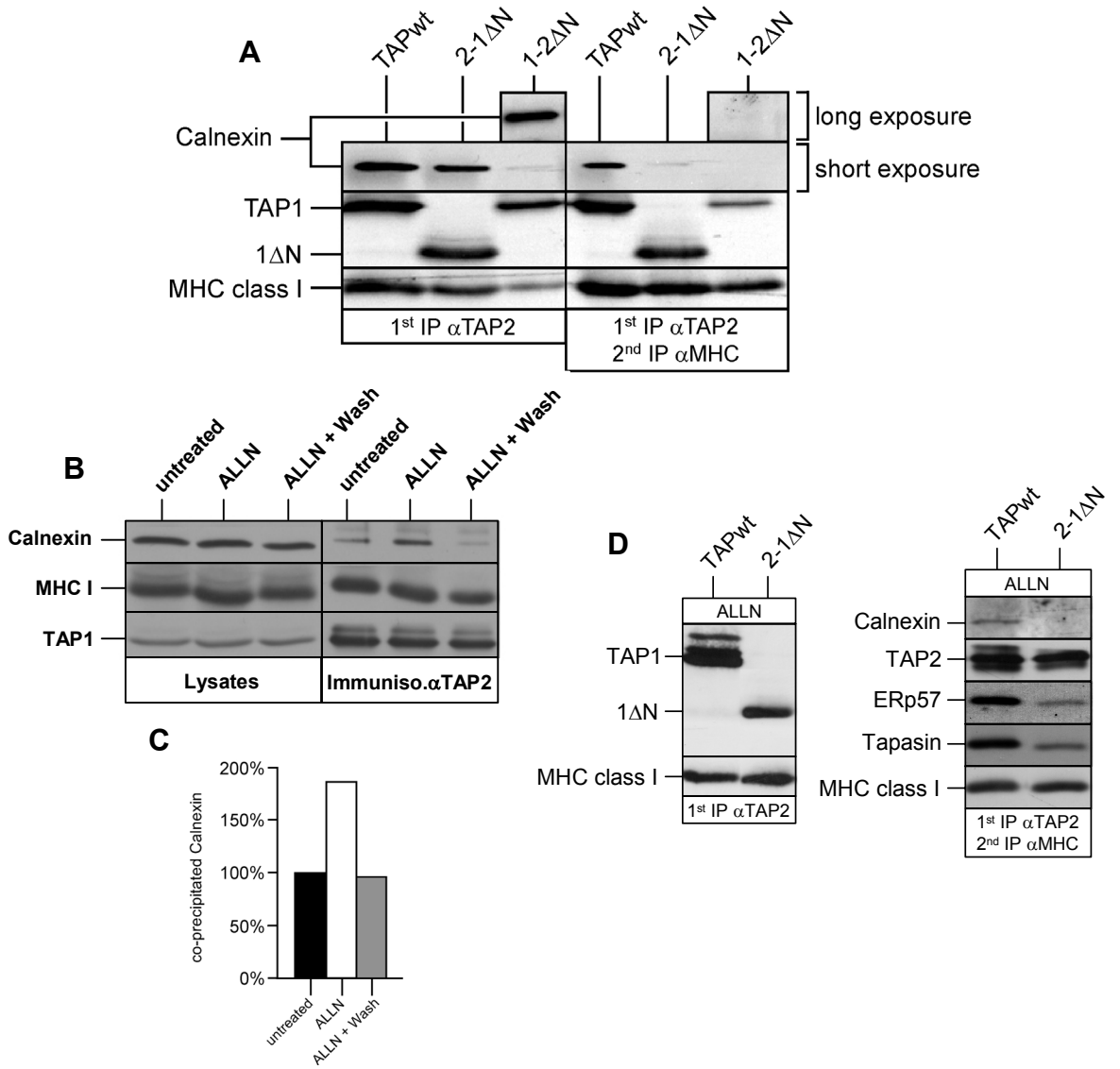

Fig. 17 **Association of calnexin with the PLC**
A) Association of calnexin with the PLC. TAP-associated proteins were immuno-isolated from digitonin lysates of cells expressing TAPwt, 2-1ΔN or 1-2ΔN, using the TAP2-specific antiserum 116/5. Bound complexes were eluted by competition with peptide, corresponding to the C-terminus of TAP2 and re-precipitated with the anti-MHC class I-specific antibody 3B10.7. SDS-PAGE-separated proteins were transferred onto nitrocellulose membrane and stained with antisera, directed against calnexin, TAP1 or MHC class I. **B)** ALLN enhances the physical interaction between calnexin and TAP. T2 transfectants were incubated in the absence or presence of the proteasome inhibitor ALLN and lysed either immediately or after removal of the inhibitor followed by overnight-incubation in ALLN-free medium. TAP2-associated proteins were isolated and analyzed by Western blot. **C)** The levels of TAP2-bound calnexin were quantitated from the Western blot shown in Fig. 17B by densitometric scanning and peak integrals, normalized for precipitated TAP1 were plotted as bar graphs. **D)** ALLN application does not induce calnexin recruitment into PLCs comprising 2-1ΔN. PLCs were isolated by consecutive immunoprecipitation form digitonin-lysates of ALLN-treated cells as in Fig. 17A. Anti-TAP2-immunoprecipitates from the first round of IP (*left panel*) or mature PLCs isolated by consecutive IP (*right panel*) were separated by SDS-PAGE and analyzed by Western Blot.

precursor complexes (that lack MHC class I) and mature loading complexes (that contain MHC class I) in the cell. Alternatively, this result might also be explained by some loss of calnexin from the PLC during the consecutive immunoprecipitation.

Nevertheless, in either case a significant fraction of TAP-MHC class I-complexes appears to be concomitantly associated with calnexin. In line with the results shown in Fig. 16 calnexin also co-precipitated with both truncation variants of the transporter. As expected (see Fig. 16), variant 2-1ΔN associated with moderately reduced levels, whereas variant 1-2ΔN bound dramatically reduced levels of the lectin chaperone when compared to TAPwt (Fig. 17A, compare lane 1, 2 and 3). (It should be noted that in this experiment the levels of MHC class I co-isolated with 1-2ΔN (Fig. 17A, lane 3) as well as the levels of TAP1 co-precipitated with MHC class I from the respective eluate (Fig. 17A, lane 6) were somewhat lower than expected. This may be caused by a reduced precipitation efficiency, a partial disassembly of the less stable loading complexes formed by 1-2ΔN or some proteolysis of precipitated proteins during the prolonged elution procedure at room temperature.) Nevertheless, the calnexin levels in mature PLCs formed by truncated TAPs were dramatically reduced (in the case of 2-1ΔN) (Fig. 17A, lane 5) or not detectable at all (in the case of 1-2ΔN) (Fig. 17A, lane 6). This indicates that the presence of the lectin chaperone and MHC class I in the loading complexes comprising 1-2ΔN or 2-1ΔN is mutually exclusive. Moreover, the distinctive interaction of calnexin with PLCs formed by truncated TAPs and TAPwt demonstrates that the observed association of the lectin chaperone with the wild-type loading complex is indeed specific. This underscores that calnexin is a regular component of the mature PLC in human cells. In addition, the findings described in this thesis show also that the wild-type transporter at steady state associates with MHC class I solely via one but not simultaneously via both N-domains (Fig. 7B, Fig. 12D, Fig. 16). Thus, in combination of these findings it is tempting to speculate that the opposing tapasin-docking site

(currently not interacting with class I) is concomitantly involved in the transient calnexin interaction (Fig. 23).

Treatment of cells with the cell-permeable proteasome inhibitor ALLN has been reported to enhance and extent the association of TAP with MHC class I molecules (Hughes et al, 1996). From these studies it had been concluded that under conditions that interfere with the availability of suitable peptide ligands in the ER lumen, empty MHC class I molecules stably accumulate in the PLC. One surprising result described above is that although both, TAP1 and TAP2 comprise a tapasin/MHC class I-docking site in their N-domain (Fig. 7 and Fig. 16) only one appears to be used for MHC class I-interaction at steady state (Fig. 7B, Fig. 12D, Fig. 16), whereas the other is occupied with calnexin (Fig. 17A and Fig. 23). Consequently, the question arises whether this organization of the loading complex indeed reflects a physical inability of class I to associate with both N-domains of TAP at the same time or if the observed steady state composition of the PLC is solely a result of the high dissociation rate of MHC class I from TAP caused by the optimal availability of peptide ligands. In the case of the latter scenario, it would be expected that under conditions where the peptide delivery into the ER is blocked (e.g. in the presence of the proteasome inhibitor ALLN), empty MHC class I molecules would displace calnexin from TAP and stably associate with both subunits of the transporter. To address this point, the cell line T2(TAPwt) was grown in the presence or absence of 250 μM ALLN for 4 hours before lysis in digitonin-containing buffer and precipitation of TAP-associated polypeptides using the TAP2-specific antiserum 116/5. Interestingly, Western blot analysis of co-isolated proteins did not show any increased levels of MHC class I bound by TAP in the presence of ALLN in three independent experiments, one of which is shown in Fig. 17B (compare lane 4 and 5), indicating that in non-treated cells all available docking-sites for MHC class I are

already saturated at steady state. Furthermore, the results shown in Fig. 17B underscore that even under conditions of extreme peptide starvation MHC class I molecules are not able to displace calnexin from TAP. This demonstrates that peptide supply does not affect the usage of tapasin-docking sites in the PLC by MHC class I. Consequently, the association of MHC class I molecules with both N-domains of TAP at the same time is a structural state that cannot be attained by the PLC. One possible explanation may be that the formation of a loading complex via one N-domain sterically hinders the formation of a corresponding complex at the docking-site in the opposing TAP subunit. Surprisingly, the calnexin signals recovered with TAP were significantly increased in the presence of the proteasome inhibitor (Fig. 17B, compare lane 4 and 5, for quantification see Fig. 17C). This is not due to an increase in overall TAP levels in the lysate as the proteasome inhibitor did not affect TAP expression (Fig. 17B). Thus, the results shown in Fig. 17B indicate that either additional calnexin molecules are recruited into the PLC upon proteasome inhibition or that the association of calnexin with the PLC is structurally stabilized in the absence of peptide supply. As the observed increase of co-isolated calnexin in the anti-TAP immunoprecipitation is not paralleled by a comparable decrease in co-isolated MHC class I it can be excluded that additional calnexin molecules occupy binding sites in the PLC that had previously been associated with class I (Fig. 17B; see also Fig. 17D, left panel). Thus, the finding that more calnexin can be co-precipitated with TAP in the presence of ALLN is more likely to reflect an enhanced physical interaction between TAP and the lectin chaperone than an increase in the total number of bound calnexin molecules per PLC. As a recent study suggests that peptide-occupation of TAP qualitatively affects its tapasin-mediated interaction with class I (Owen et al, 2001), a similar view may be applied for the tapasin-mediated interaction of calnexin with the transporter. It is plausible to assume that in the

presence of ALLN an intermediate of the PLC is trapped, which corresponds to the state where TAP is not associated with cytosolic peptide substrates. Possibly, this intermediate is characterized by a higher affinity for the lectin chaperone. One prediction that such a model would make, is that the enhanced physical interaction between TAP and calnexin observed under conditions of peptide starvation should become reversed upon the restoration of the peptide supply into the ER. To validate this, T2(TAPwt) cells were treated with 250 μM ALLN for 4 hours, followed by extensive washing for removal of the proteasome inhibitor and subsequent over-night incubation in ALLN-free medium. 24h later the cells were lysed and TAP-associated polypeptides were isolated with the TAP2-specific antiserum 116/5. As expected Western blot analysis of the co-isolated proteins showed that the signals for calnexin recovered with anti-TAP2 antiserum under these conditions returned to levels observed for non-treated cells (Fig. 17B and Fig. 17C). To confirm the results shown in Fig. 17B mature MHC class I-containing loading complexes were isolated by consecutive immunoprecipitation (as for Fig. 17A) from ALLN-treated T2(TAPwt) or T2(2-1ΔN) cells. Consistent with the finding that in the presence of ALLN MHC class I does not displace calnexin from the wild-type PLC, TAPwt and 2-1ΔN bound comparable levels of class I even when the proteasome inhibitor was applied (Fig. 17D, left panel). Furthermore, in line with the idea that binding of calnexin and MHC class I to the truncated transporters is mutually exclusive mature loading complexes formed by 2-1ΔN did not show any detectable association with the lectin chaperone (Fig. 17D, right panel). In contrast, calnexin was clearly present in PLCs formed by TAPwt (Fig. 17D, right panel). Finally, the results shown in Fig. 17D provide further evidence that PLCs comprising 2-1ΔN contain only one of the two docking sites for the recruitment of tapasin and ERp57. Nevertheless, as expected from the results

depicted in Fig. 7B and Fig. 16 MHC class I associates comparably with mature PLCs formed by wild-type TAP or 2-1ΔN.

Taken together, the results shown in Fig. 17 support a model of the PLC wherein one N-domain of TAP is involved in tapasin-mediated interaction with MHC class I, whereas the opposing N-domain is simultaneously involved in the transient tapasin-mediated interaction with calnexin (Fig. 23).

5.7 Calnexin is not essential for recruitment of ERp57 into the loading complex or for the quality control of antigen presentation

The results depicted in Fig. 16 suggest that the truncation variant 1-2ΔN associates with significantly lower levels of ERp57, calreticulin and calnexin than the wild-type transporter. Since ERp57 is believed to become recruited to its substrate proteins via binding to one of the two lectin chaperones (Ellgaard et al, 2004) the question arose whether calnexin is responsible for the insertion of the oxidoreductase into the PLC. To address this question, TAP-associated proteins were co-isolated with the monoclonal TAP1-specific antibody 148.3 from digitonin lysates of the calnexin-proficient human T cell leukaemia cell line CEM or its calnexin-deficient derivative CEM-NKR (Fig. 18A). Precipitates were analyzed by Western blot using antibodies targeted against TAP1, calreticulin, tapasin and ERp57 (Fig. 18B). Interestingly, PLCs derived from CEM-NKR cells displayed no reduction in ERp57 association, indicating that calnexin is not essential for recruitment of ERp57 into the PLC.

The results depicted in Fig. 17A and Fig. 17D suggest that calnexin and MHC class I do not concomitantly associate with the same TAP subunit in the wild-type loading complex. This might argue against a direct influence of calnexin on the loading of MHC class I molecules. However, as roles for the other components of the PLC in the quality control of antigen presentation have been proposed (Dick et al, 2002; Gao

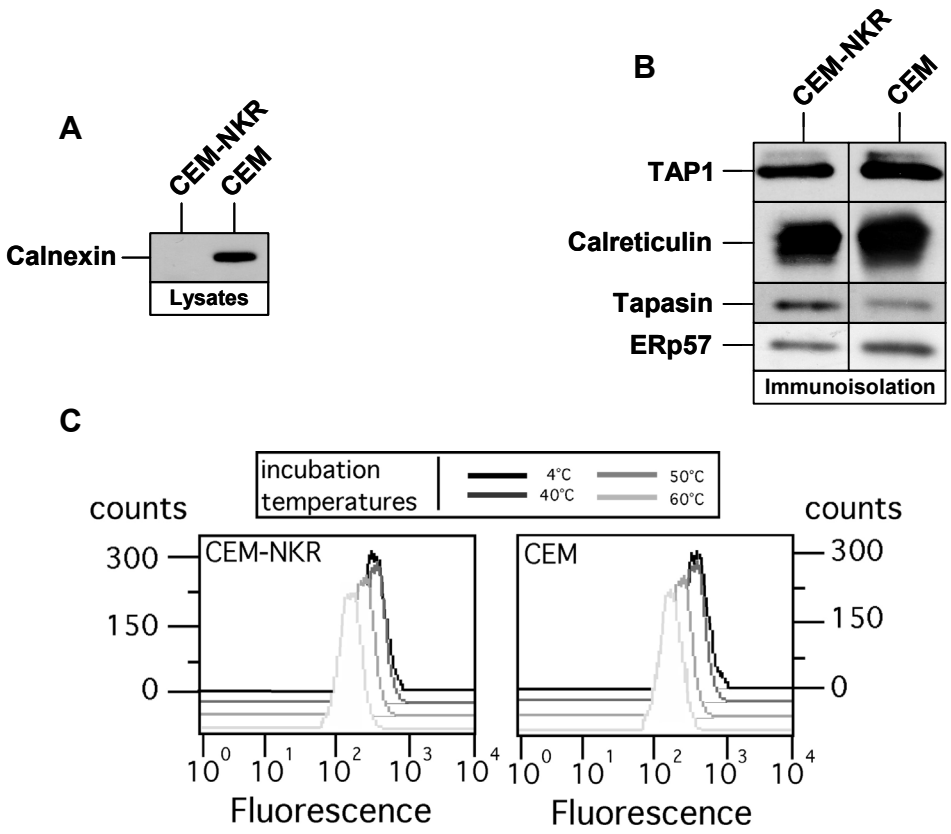

Fig. 18 **Characterization of the peptide loading complex and MHC class I stability in cells lacking calnexin**
A) Expression of calnexin. Cell lysates were analyzed in a Western blot probed for calnexin. **B)** Comparison of PLC formation. Immunoblots of SDS-PAGE-separated proteins from αTAP1 precipitates were probed for TAP1, tapasin, ERp57 and calreticulin. **C)** Thermostability of intracellular MHC class I in CEM and CEM-NKR cells. Cells were collected in PBS / 0,1% NaN_3 before incubation at 4, 40, 50 or 60 °C for 10 min. Subsequently, cells were fixated, permeabilized and stained with the monoclonal αMHC class I antibody w6/32. After incubation with FITC-labeled secondary antibody MHC class I levels were determined by flow cytometry.

et al, 2002; Tan et al, 2002; Williams et al, 2002), it was analyzed whether or not calnexin is a part of the machinery that directs the generation of stable MHC class I molecules in the ER. Therefore, the thermostability of intracellular MHC class I molecules in calnexin-deficient CEM-NKR cells and their calnexin-proficient counterparts CEM was examined by flow cytometry (Fig. 18C). For these studies the monoclonal anti-MHC class I antibody w6/32 (Barnstable et al, 1978) was employed, which recognizes β_2m-associated HLA-A, HLA-B and HLA-C molecules. Fig. 18C shows that the total MHC class I population in calnexin-positive CEM cells displayed a higher thermolability than HLA-B5 in T2(TAPwt) cells (Fig. 15A). Allele-specific

differences in MHC class I stability (Zernich et al, 2004) are likely to account for this. However, no further reduction of MHC class I thermostability could be observed in calnexin-deficient CEM-NKR cells, suggesting that calnexin has at least no significant influence on the stability of the MHC class I alleles expressed in CEM cells.

5.8 High level MHC class I surface expression in the cell line T2(1-2ΔN) depends on the proteolytical activity of proprotein convertases

The data regarding the stability of MHC class I in the established cell lines (Fig. 14 and Fig. 15) showed that the fraction of surface HLA-B5 in the transfectant T2(1-2ΔN) was significantly more thermoresistant than the intracellular population (Fig. 15A). Since the stability of this class I allele improved over time in a post-ER compartment (Fig. 15C) the question arose, which cellular mechanism was responsible for the observed effect. To address this point, it was first determined whether the transfectants expressing 1-2ΔN gained stability of surface HLA-B5 complexes by an increased rate of endocytic recycling and re-loading of MHC class I with peptides generated by endosomal processing. Therefore, the cell lines T2(TAPwt), T2(1-2ΔN) or T2(2-1ΔN) were grown in the presence of primaquine (Fig. 19, grey bars) or NH_4Cl (Fig. 19, white bars) before HLA-B5 surface levels were analyzed by flow cytometry. Primaquine is a drug that causes intracellular accumulation of endocytosed proteins including MHC class I molecules through a block in endocytic recycling (Reid et al, 1990) whereas NH_4Cl inhibits endosomal processing (Ohkuma et al, 1978). Interestingly, the different transfectants showed almost identical reductions in the surface presentation of HLA-B5 in the presence of the lysosomotropic reagents (up to 25% for primaquine, see Fig. 19). This indicates that the analyzed cell lines do not markedly differ in the endocytic recycling and processing of surface MHC class I.

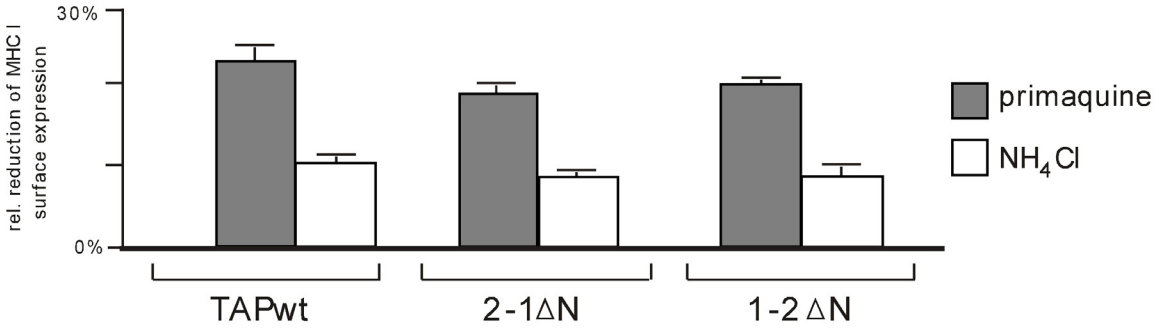

Fig. 19 **Endocytic recycling and endosomal processing of MHC class I**
Expression of surface HLA-B5 in the presence or absence of primaquine or NH_4Cl was analyzed by flow cytometry. Results are plotted as percentage of reduction of the MFI compared with the MFI of the control experiments.

Stryhn and co-workers had proposed that suboptimally loaded MHC class I molecules, which are exported from the ER, may exchange peptide ligands with low affinity in the acidic milieu of the late secretory compartments (Stryhn et al, 1996). Furthermore, proprotein convertases (PCs) (Seidah et al, 1998), some of which are known to cycle between the *trans*-Golgi network (TGN), plasma membrane and endosomes (Rockwell et al, 2004; Seidah et al, 1998), have been reported to be involved in the generation of peptide antigens for MHC class I (Gil-Torregrosa et al, 2000; Zhang et al, 2001). Thus, one promising hypothesis was that the improvement of MHC class I stability in the cell line T2(1-2ΔN), which occurred along the exocytic route reflected re-loading of unstable MHC class I molecules in the TGN with proprotein convertase-generated peptides (see working model in Fig. 24). The human genome encodes at least seven highly related members of the subtilisin/kexin-like prohormone convertase family of serine proteases (Seidah et al, 1998). Two of these, namely PC1 and PC2 are exclusively expressed in endocrine and neuroendocrine cells (Bennett et al, 1992; Seidah et al, 1990; Seidah et al, 1992), whereas PC4 has been reported to be expressed solely in testis (Nakayama et al, 1992). However, the remaining four members of the proprotein convertase family, namely furin (Roebroek et al, 1986), PC5 (Mercure et al, 1996), PC7

(Bruzzaniti et al, 1996) and PACE4 (Kiefer et al, 1991) are known to display a widespread tissue distribution (Bruzzaniti et al, 1996; Hatsuzawa et al, 1990; Kiefer et al, 1991; Lusson et al, 1993; Mercure et al, 1996; Seidah et al, 1996; Tsuji et al, 1994). As T2 cells derive from a hybrid ancestor (T1) generated by the fusion of a B-lymphoblastoid cell line (LCL 721.174) with a T- lymphoblastoid cell line (CEM) (Salter et al, 1985), it is interesting to note that several B-cell lines were also reported to express furin and PC7 (Zarkik et al, 1997) while some T-cell lines additionally harbour PC5 (Decroly et al, 1996; Decroly et al, 1997). To investigate whether T2 cells express any of the proprotein convertases displaying broad tissue distribution RT-PCR was performed using primers specific for furin, PC5, PC7 or PACE4 (Fig. 20A). In this experiment the human hepatoma cell line HepG2 served as a positive control as previous studies had shown that this cell line expresses several PC members (Imamaki et al, 1999). mRNA prepared from both cell lines was kindly provided by C. Bekpen. As depicted in Fig. 20A transcripts of all four proprotein convertases could be readily detected in HepG2 cells, whereas the T2 cell line expressed solely furin and PC7. To additionally confirm at the protein level the presence of these two proprotein convertases in T2 cells respective lysates were separated by SDS-PAGE and analyzed by Western Blot using PC-specific antisera. As shown in Fig. 20B, bands corresponding to furin and PC7 could be detected in T2 cell lysates at the expected size of approx. 92 kDa. To further analyze the subcellular distribution of furin and PC7 in T2 cell lines immunofluorescence studies were undertaken. The fluorescence signal measured for PC7 was found to be most intense in a juxta-nuclear region that seemed to consist of several membranous subcompartments (Fig. 20C-d and Fig. 20C-e). Additionally, some cells displayed strong anti-PC7 immunoreactivity associated with vesicular structures immediately beneath the plasma membrane (Fig. 20C-f). This is consistent with earlier

descriptions of PC7 as a proprotein convertase distributed within the TGN (Van de Loo et al, 1997) or post-TGN vesicles (Wouters et al, 1998). Furin was also found to

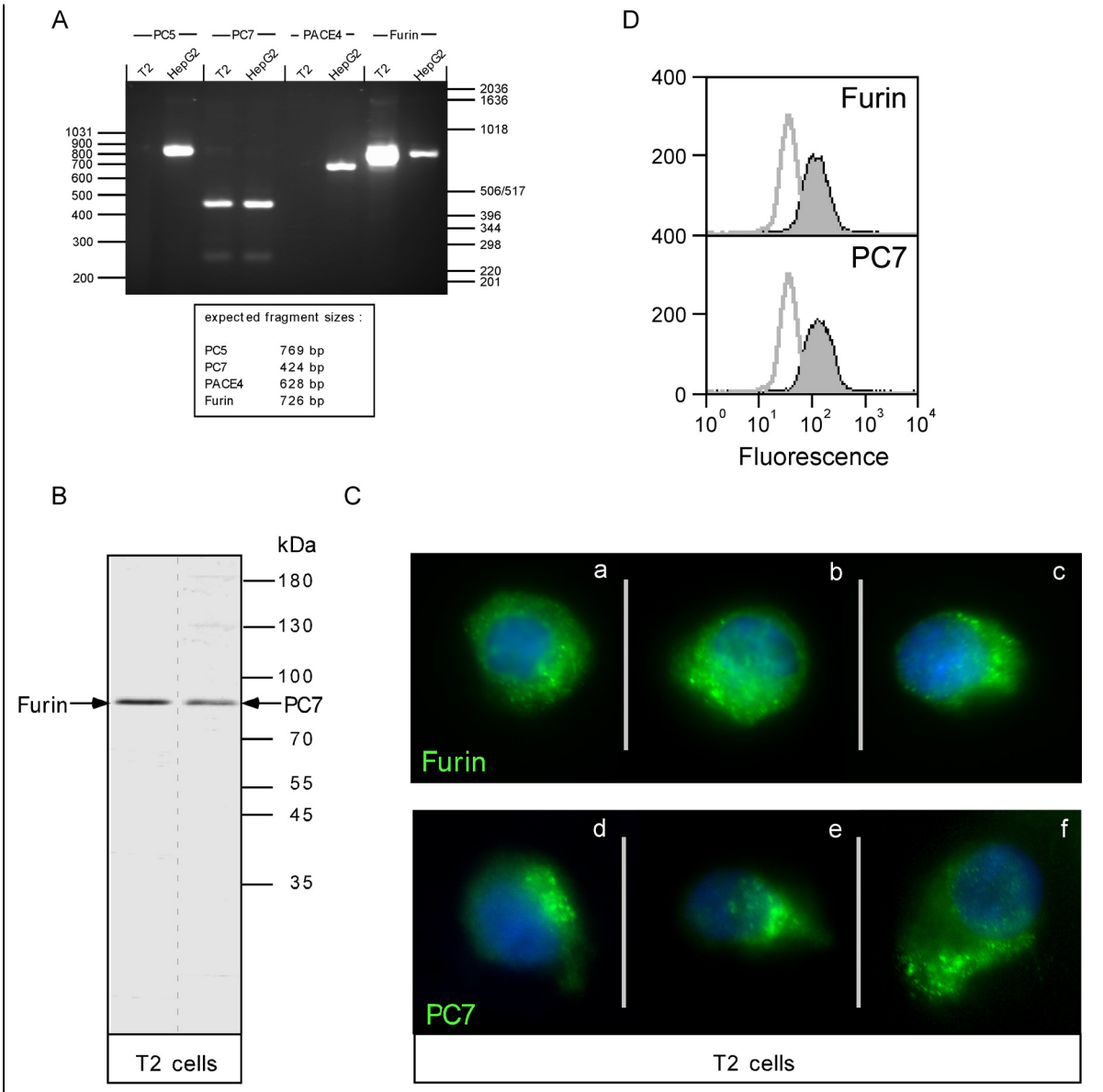

Fig. 20 **Expression of proprotein convertases in T2 cells**
(A) Detection of proprotein convertase transcripts by RT-PCR. mRNA derived from T2 or HepG2 cells (kindly provided by C. Bekpen) served as a template for RT-PCR using proprotein convertase-specific primers. **(B)** Expression of furin and PC7 in T2 cells. Lysates of T2 cells were analyzed by Western blot using the furin-specific antiserum anti-Furin or the PC7-specific antiserum KP-1. **(C)** Subcellular localization of proprotein convertases in T2 cells. Cells were fixated, permeabilized and stained with proprotein convertase specific antisera followed by Alexa 488-conjugated secondary antibody. Subsequently cells were analyzed using a Zeiss Axioplan II fluorescence microscope. **(D)** Specificity of furin and PC7 stainings. T2 cells were fixated, permeabilized and stained with proprotein convertase specific antisera. After incubation with Alexa 488-labeled secondary antibody intracellular proprotein convertase levels were determined by flow cytometry (filled histograms). Unspecific background staining was analyzed by incubation with secondary antibody alone (non-filled histograms).

be partially distributed in Golgi-like structures (Fig. 20C-c). However, anti-furin immunoreactivity was clearly most prominent in small dots that appeared to be spread throughout the cell (Fig. 20C-a and Fig. 20C-b). This pattern strongly resembles the characteristic staining pattern of the endosomal system, suggesting that furin may be predominantly localized to endosomes in T2 cells. To confirm the specificity of the immunofluorescent stainings displayed in Fig. 20C flow cytometry analysis of proprotein convertase expression using the same combination of primary and secondary antibodies was performed (Fig. 20D). To investigate whether the high level of surface MHC class I expression in the cell line T2(1-2ΔN) depends on the activity of proprotein convertases, surface MHC class I-recovery experiments in the presence of increasing concentrations of the PC-specific, cell permeable inhibitor hexa-D-arginine (HDA) (Fig. 21) were performed (Cameron et al, 2000; Sarac et al, 2002). In T2 cells expressing wild-type TAP and 2-1ΔN, the recovery of surface MHC class I was not or only slightly affected (Fig. 21). This suggests that in these two cell lines the proprotein convertase-dependent pathways play only a minor role in the generation of MHC class I ligands. However, transfectants expressing variant 1-2ΔN showed in the presence of HDA a significant and concentration-dependent reduction (up to 45% at 100 µM HDA) of surface MHC class I suggesting that a substantial amount of HLA-B5 antigens originates from proteolytic products generated by furin or related PCs (Fig. 21).

Thus, it seems that in a situation where the quality control in the loading complex fails a post-ER mechanism that depends on proprotein convertase-activity can rescue stable surface expression of MHC class I molecules (Fig. 24).

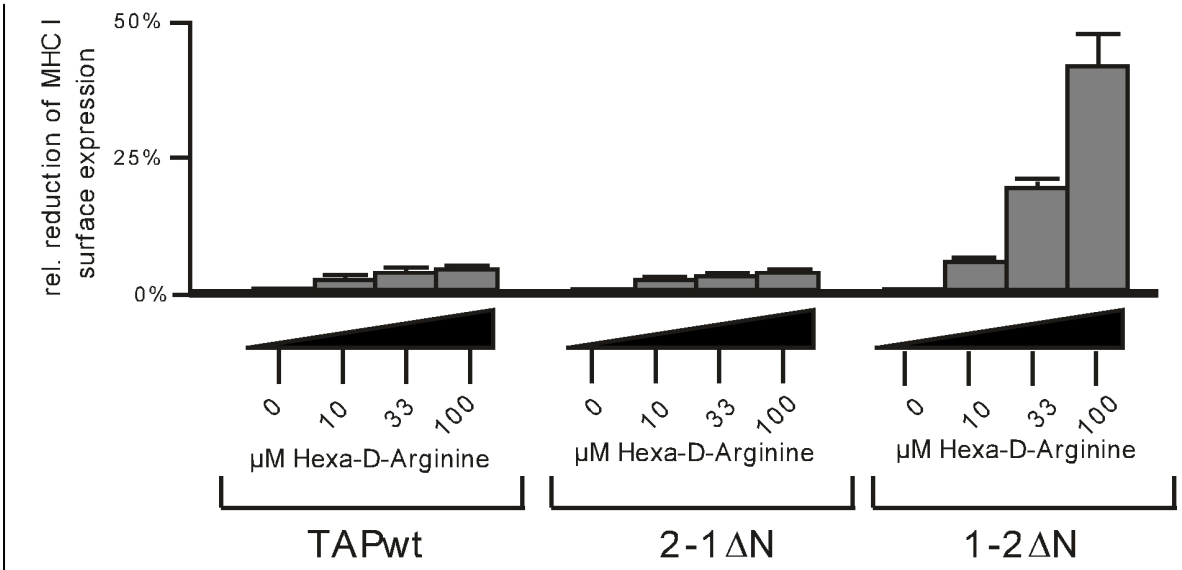

Fig. 21 **Proprotein convertase-dependence of MHC class I surface expression**
Proprotein convertase-dependence of MHC class I surface expression. Cells were acid-washed to remove assembled surface MHC class I and incubated for 14h in the presence of increasing concentrations of the proprotein convertase inhibitor hexa-D-arginine. Subsequently, cells were stained with monoclonal antibody 4E followed by FITC-labeled secondary antibody and surface expression of HLA-B5 was determined by flow cytometry. Surface MHC class I staining in the presence of hexa-D-arginine is plotted as percentage of reduction of the MFI compared with the MFI of the control experiments.

5.9 Proprotein convertase expression is differentially regulated by interferons at the transcriptional level

The expression of diverse immune-relevant genes is regulated by interferons, among them many that play a role in the MHC class I antigen presentation pathway (Boehm et al, 1997). As interferons become secreted in the context of an acute infection (Boehm et al, 1997) the upregulation of a gene in response to these cytokines has in the past been regarded as indicative of an immune-related function. However, several abundant proteins that serve for housekeeping functions in the cell, but additionally play an important role in antigen presentation (e.g. the constitutive proteasome, calnexin or calreticulin) have not been reported to be induced by interferons. It is noted that the regulation of proprotein convertase expression in virally infected cells may be of particular interest as this class of furin-related proteases besides a possible role in antigen presentation (Fig. 21) is required for the proteolytic maturation of numberless viral proteins, including those of human

immunodeficiency virus (HIV) (Vollenweider et al., 1996), hepatitis B virus (HBV) (Messageot et al, 2003), severe acute respiratory syndrome coronavirus (SARS) (Bergeron et al, 2005), human respiratory syncytial virus (HRSV) (Gonzalez-Reyes et al, 2001), influenza A Hong Kong virus (HKV) (Basak et al, 2001), measles virus (Bolt et al, 1998), human cytomegalovirus (HCMV) (Vey et al, 1995) and ebola virus (Basak et al, 2001). Thus, in view of the finding that under certain conditions proprotein convertases contribute peptides to the MHC class I antigen presentation pathway (Fig. 21) the up- or downregulation of proprotein convertases in the context of a viral infection may turn out to be a two-edged weapon. To determine whether furin or PC7 are regulated at the transcriptional level by interferon-γ the human cell lines T2 (a lymphoblastoid cell line), THP-1 (a monocyte-derived cell line) or HeLa (a cervical carcinoma cell line) were stimulated with 200 U/ml interferon-γ for 24h before mRNA was prepared. This mRNA, which was kindly provided by C. Bekpen, served as a template in an RT-PCR in combination with furin- or PC7-specific primers. To control for the equal amount and quality of the mRNA derived from uninduced or induced cells, RT-PCR was performed with primers specific for the interferon-unresponsive (Baldeon et al, 1998) housekeeping gene GAPDH (Hanauer et al, 1984). Additionally, to validate that the induction with the indicated cytokine was successful, RT-PCR was carried out using primers specific for the genes hGBP1 (Cheng et al, 1991) or MxA (Aebi et al, 1989), both of which encode immune-relevant GTPases (MacMicking et al, 2004) that have been reported to be highly upregulated upon interferon induction (Aebi et al, 1989; Andrews et al, 2002; Cheng et al, 1986). As shown in Fig. 22 the expression of PC7 was not or only moderately affected at the transcriptional level in all cell lines tested. However, furin transcripts were despite their apparent presence in uninduced cells only barely detectable (in T2 cells) or not detectable at all (in THP-1 and HeLa cells) in cells stimulated with interferon-γ (Fig.

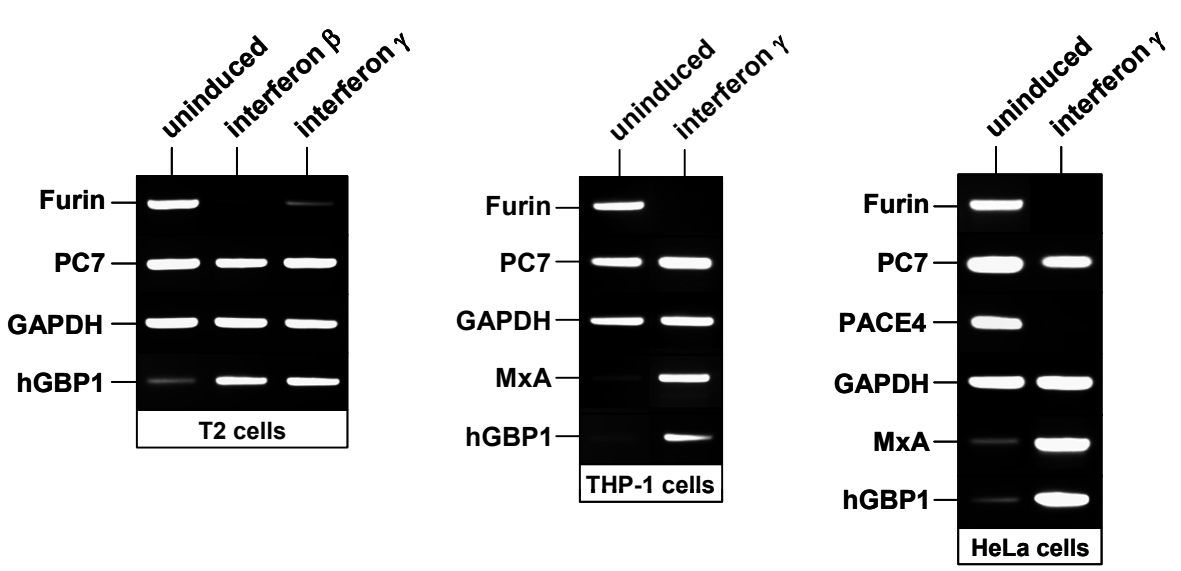

Fig. 22 **Interferon-regulation of proprotein convertase expression**
The human cell lines T2 (lymphoblastoid), THP-1 (monocyte-derived) or HeLa (cervical carcinoma) were stimulated for 24h with interferon-γ or interferon-β at a concentration of 200 U/ml or 2000 U/ml, respectively. The mRNA that was subsequently prepared from the indicated cell lines was kindly provided by C. Bekpen for this experiment. RT-PCR was performed using this mRNA as a template in combination with proprotein convertase-specific primers. Equal amount and quality of mRNA isolated from induced and uninduced cells was controlled by RT-PCR using primers specific for GAPDH, an interferon-unresponsive housekeeping gene. The induction by interferons was controlled by RT-PCR using primers specific for the interferon-regulated genes hGBP1 or MxA.

22). This clearly indicates that furin is strongly (and in some cell lines completely) downregulated at the transcriptional level in response to this cytokine. Hence, it is tempting to speculate that during most viral infections PC7 is the proprotein convertase that eventually rescues unstable MHC class I molecules in the *trans*-Golgi-network whereas the expression of furin is repressed to impede viral replication. Interestingly, PACE-4 was also found to be strongly downregulated in HeLa cells in response to interferon-γ (Fig. 22). Thus, one may speculate that in the case of an infection the synthesis of a whole battery of enzymes, all characterized by overlapping cleavage-specificities and all able to promote the cleavage-activation of viral proteins (with varying efficiency) is switched off. Most interestingly, the treatment with interferon-β lead also to a dramatic decrease in the levels of furin transcripts in T2 cells suggesting that type I (α/β) and type II (γ) interferons regulate the synthesis

of proprotein convertases in a coordinate and (possibly) synergistic manner as described in the case of many other genes (Boehm et al, 1997).

Taken together, in diverse cell lines the expression of proprotein convertases at the transcriptional level appears to be distinctively modulated in response to interferons. Additionally, the results shown in Fig. 22 suggest that even under conditions of interferon-mediated downregulation of certain proprotein convertases, persistent expression of PC7 may allow for the continuing generation of MHC class I antigens in the *trans*-Golgi network.

6. Discussion

6.1 At steady-state TAP does not simultaneously associate with MHC class I via both N-domains

To investigate the function of the N-terminal tapasin-binding sites in TAP1 and TAP2 for the proper processing of MHC class I molecules, TAP variants lacking the N-domain in TAP1 (2-1ΔN) or TAP2 (1-2ΔN) were stably expressed in the human TAP-negative cell line T2 (Fig. 5B). Both mutant transporters clearly bound peptides (Fig. 6A) and displayed normal substrate translocation activity when compared to the wild-type transporter (Fig. 6B). Consequently, MHC class I molecules were efficiently supplied with ligands in T2 transfectants expressing TAPwt, 1-2ΔN or 2-1ΔN. This was reflected in almost identical dissociation kinetics of MHC class I from the PLC (Fig. 7B), comparable ER-export rates of HLA-B5 (Fig. 8A and Fig. 15C, closed symbols), similar time-courses of HLA-B5 surface recovery (Fig. 8B) and equal steady state surface expression of both, HLA-B5 (Fig. 8C) and HLA-A2 (data not shown) in the established cell lines.

Consistent with the results of others (Koch et al, 2004) a transporter variant lacking both N-regions (TD1ΔN/2ΔN) was shown to be completely excluded from the PLC (Fig. 11A), whereas a similar construct retaining the N-domain in TAP1 readily bound tapasin (Fig. 11A). This indicates that the N-terminus of TAP1 indeed acts as a docking site for the loading complex. Conversely, the capability of the heterodimeric variant 2-1ΔN to form a PLC (Fig. 7A, Fig. 16 and Fig. 17D) implies that also the N-domain in TAP2 acts as a tapasin binding module. In accordance with the above mentioned findings pulse-chase experiments showed that both N-domains are able to form functional loading complexes, from which MHC class I molecules dissociate following the acquisition of peptide cargo (Fig. 7B). Consequently, it is attractive to

speculate that the two TAP subunits in the wild-type transporter normally alternate with each other in the tapasin-dependent recruitment of newly synthesized MHC class I molecules. As expected from the finding that both N-domains can act as docking sites for components of the PLC, the wild-type transporter appeared to associate with significantly higher levels of tapasin (and ERp57) than the two truncated transporters 1-2ΔN and 2-1ΔN (Fig. 16 and Fig. 17D). Hence, it is tempting to speculate that in the case of TAPwt both N-domains simultaneously interact with these chaperones (Fig. 23). Surprisingly, the situation was found to be completely

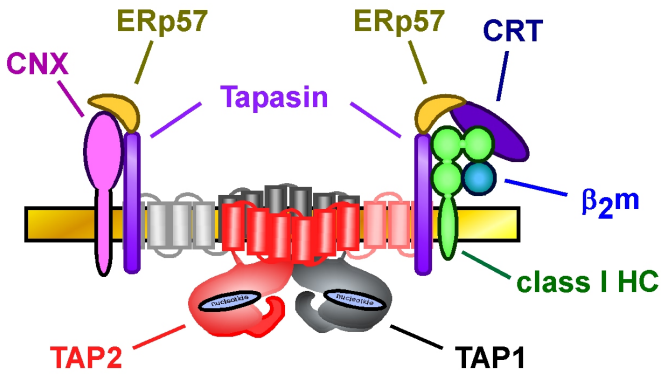

Fig. 23 **Current working-model for the peptide loading complex**
The results presented in this thesis argue for an asymmetric peptide loading complex, in which one N-domain of the central TAP molecule is involved in the tapasin-mediated MHC class I-interaction, whereas the other associates with calnexin (CNX). A consequence of the postulated model is that both N-domains are simultaneously associated with tapasin and ERp57 at steady state. In contrast, the calreticulin (CRT)/MHC class I-subcomplex as well as calnexin are at any given time solely bound by one of the two TAP subunits.

different for MHC class I and calreticulin. Western blot analysis showed that both polypeptides were co-isolated in almost identical amounts with TAPwt, 2-1ΔN and even the TAP single chain 1ΔN (Fig. 12D, Fig. 16 and Fig. 17D). In addition also 1-2ΔN associated comparably with MHC class I molecules (Fig. 16). In accordance with these results, pulse-chase experiments displayed very similar kinetics for the release of MHC class I from wild-type TAP and the two truncated transporter variants (Fig. 7B). This finding and the observation that similar amounts of radiolabeled MHC class I co-precipitated with the three TAP variants at any time point of the pulse-

chase experiment (Fig. 7B) indicates that during the chase period comparable amounts of newly synthesized radiolabeled MHC class I passed through the three different loading complexes. Thus, although wild-type TAP has the capability to recruit MHC class I via both subunits, it seems that in the functional PLC at any given time only one of two N-domains is actively involved in the tapasin-mediated recruitment, loading and release of newly synthesized MHC class I (Fig. 23). Interestingly, this holds true even under conditions of extreme peptide starvation in the presence of the proteasome inhibitor ALLN (Fig. 17D, left panel). It should be noted that these findings clearly contrast with the initial view on the PLC that speculated simultaneous binding of class I to both TAP subunits would favour the association of multiple MHC class I alleles with the transporter, thereby increasing the probability that a translocated peptide gains immediate access to an appropriate allele (Ortmann et al, 1997). This model founded on an early study regarding immunoisolated loading complexes, which suggested that one TAP heterodimer binds four tapasin and four MHC class I molecules at steady state (Ortmann et al, 1997). However, as a recent report demonstrated that tapasin does not form dimers, trimers or tetramers (Bangia et al, 2005) the predicted stoichiometry would demand that four individual tapasin/MHC class I-complexes simultaneously interact with the transporter. Furthermore, taking into account the finding that at steady state solely one TAP subunit associates with MHC class I (Fig. 7B, Fig. 16 and Fig. 17D), a model would follow that claims that each of the two N-domains (comprising solely 3 or 4 transmembrane helices, respectively) shares the capability to interact with four independent tapasin-MHC class I-complexes at the same time. Although the data presented in this thesis does not formally exclude such a PLC, it is not considered likely either. In better accordance with the results described here, another group investigated the composition of the human TAP-complex by velocity sedimentation

analysis and provided evidence for a TAP-associated loading complex with a lower number of tapasin and MHC class I molecules (Li et al, 1997). Nevertheless, the question remains why the transporter does not simultaneously use its N-domains for recruitment of MHC class I as both TAP subunits appear to be occupied with tapasin at steady state (Fig. 16 and Fig. 17D). One possible explanation may be that several viruses considerably interfere with the proper function of the transporter, which must be regarded as a bottleneck of the MHC class I antigen presentation pathway (Hewitt et al, 2003; Lybarger et al, 2005). Thus, under conditions of a viral infection one could speculate that it would be beneficial for the cell to increase the number of transporter molecules in order to titrate away viral effectors or at least to temporally defer a complete viral block of peptide delivery into the ER. This would be consistent with the induction of TAP by the inflammatory cytokine interferon-γ, which is secreted by T cells or NK cells in response to an acute infection (Boehm et al, 1997). Following this argument, it may be a more promising strategy for the cellular immune system to provide a huge number of TAP molecules each associated with one (or a small number of) MHC class I alleles than to assemble PLCs that comprise a high number of MHC class I alleles bound by a single TAP molecule that in the case of its inhibition would render all bound MHC class I molecules peptide-starved and thus useless for the immune response.

6.2 Calnexin is a component of the mature human peptide loading complex

Since several years it is known that the lectin chaperone calnexin associates with the loading complex in murine cells (Suh et al, 1996). However, a corresponding interaction in the human system has despite some efforts not been detected yet (Diedrich et al, 2001). Instead, as a final step of the PLC formation in human cells

calnexin was reported to become released from the so-called precursor complex rapidly upon (or concomitantly with) the insertion of MHC class I and calreticulin (Fig. 4) (Diedrich et al, 2001). Consequently, the question arises whether the reproducible detection of calnexin in the mature PLCs of the transfectant T2(TAPwt) (Fig. 17A and Fig. 17D) reflects indeed a naturally occurring interaction in human cells or is solely due to the heterologous expression of rodent TAP. The latter possibility is unlikely as several lines of evidence point towards a role for tapasin, but not for TAP in the recruitment of calnexin into the PLC / precursor complex. First, tapasin was shown to retain the stable association with calnexin and ERp57 after cell lysis in Triton X-100, a detergent which is known to release TAP from the other components of the PLC (Diedrich et al, 2001). This indicates that TAP is not required to maintain the association of calnexin with tapasin and the oxidoreductase. Second, tapasin is capable of forming complexes with calnexin in TAP-negative T2 cells (data not shown), demonstrating that the peptide transporter is not essential to allow for an interaction between the two chaperones. Hence, these results suggest that tapasin, but not TAP directly recruits calnexin into the PLC. At present, one can only speculate why Diedrich and colleges did not detect calnexin in the human loading complex (Diedrich et al, 2001). Hitherto, it cannot be excluded that interactions among individual PLC components vary somewhat depending on the MHC class I alleles expressed in the cell as suggested by recent results of one group (Paquet et al, 2002). Thus, different class I alleles may reside in loading complexes with different compositions. However, it is more likely that calnexin-binding to the PLC was overlooked as its interaction with the PLC is presumably labile and thus easily broken. Surprisingly, the consecutive immunoprecipitation studies depicted in Fig. 17A and Fig. 17D showed that the simultaneous association of TAP with calnexin and MHC class I is solely observed in the case of TAPwt, but not in the case of the

truncation variants 1-2ΔN and 2-1ΔN. As the two latter have not generally lost the ability to interact with calnexin (Fig. 16 and Fig. 17A) it must be inferred that both interactions are mutually exclusive in the truncated TAPs. Thus, calnexin-bound TAP-complexes formed by 1-2ΔN and 2-1ΔN are with regard to their molecular composition reminiscent of the precursor complexes postulated by Diedrich and colleges (Diedrich et al, 2001). Consequently, the results presented in Fig. 17A and 17D do not rule out the existence of precursor complexes in human cells. Rather, it is possible that all docking-sites in the PLC that are occupied by MHC class I, were previously associated with calnexin. Hence, a model can be predicted, in which the docking-sites of the TAP-associated loading complex sequentially switch between two states: an MHC class I-associated loading state followed by a calnexin-associated regeneration or maintenance state (see later). Importantly, the calnexin-binding properties of the TAP truncation variants imply that the interaction of the lectin chaperone with the wild-type transporter occurs via that N-domain, which is currently not involved in the binding of MHC class I (see model of the PLC in Fig. 23). Interestingly, in the murine system the binding of peptide was shown to release MHC class I molecules from TAP but not from calnexin. From this result it was speculated that calnexin would ultimately determine whether a loaded MHC class I molecule becomes exported from the ER or not (Suh et al, 1996). Such a role for calnexin in the retention of murine class I-peptide complexes is consistent with a decelerated rate of egress reported for the assembled mouse-derived MHC class I allele H2-K^b from the ER when expressed in insect cells in combination with calnexin (Vassilakos et al, 1996). Nevertheless, the finding that in murine cells intact MHC class I-calnexin complexes become displaced from TAP upon peptide binding, suggests that here calnexin binds to the same TAP subunit as class I, making it plausible to speculate that the calnexin interaction described for the murine PLC (Suh et al, 1996) is

different from that described for the human PLC in this thesis. In line with this, no role for calnexin in the retention of assembled MHC class I molecules in human cells was found yet (Prasad et al, 1998; Scott et al, 1995). However, up to now it cannot be excluded that a concerted release of calnexin and peptide-loaded MHC class I from the two opposing N-domains in the human PLC is followed by an association of both components. Hitherto, it can only be speculated what the function of calnexin in the mature PLC may be. The immunoprecipitation study shown in Fig. 18B rules out that calnexin is essential for the recruitment of ERp57 into the loading complex. Thus, as calreticulin was also shown to be dispensable for the proper insertion of the oxidoreductase into the PLC (Gao et al, 2002) it is likely that either both lectin chaperones contribute to the incorporation of ERp57 in a redundant manner or that ERp57 is directly recruited by tapasin, to which it forms a disulfide bridge (Dick et al, 2002). Furthermore, calnexin was shown to be clearly not essential for peptide-loading, ER-export or surface expression of MHC class I as determined in the calnexin-deficient cell line CEM-NKR (Howell et al, 1985; Prasad et al, 1998; Scott et al, 1995; data not shown). Typically calnexin is known to play an important role in the folding pathway of glycosylated proteins in the ER (Fig. 3) (High et al, 2000). In addition, it can act as a molecular chaperone also on several non-glycosylated substrates (Ihara et al, 1999). Therefore, one promising hypothesis may be that the function of calnexin in the PLC is to maintain (and perhaps protect) the free MHC class I-docking site in a state that is competent for class I interaction. This would be in line with the finding that a treatment with ALLN enhances the physical interaction of calnexin with the PLC. (As this drug blocks the generation of peptides by cellular proteases its application may mimic the state of a viral infection, which is often characterized by reduced delivery of peptides into the ER through inhibition of TAP or the proteasome.) Presumably, such a function for calnexin would be dispensable and

hence difficult to observe in a non-infected healthy cell. However, it could become important in the context of a pathogenic challenge as several viruses interfere with the formation or the function of the PLC (Hewitt et al, 2003; Lybarger et al, 2005). One example for such a viral subversion strategy targeting the loading complex is the adenovirus protein E19 that binds to both, MHC class I and TAP in order to inhibit the interaction of both proteins (Bennett et al, 1999). Another example is the γ-herpesvirus 68 (HV68) protein mK3 that associates with the TAP-tapasin-complex where it presumably awaits the entry of newly synthesized class I chains, on which it acts as a ubiquitin ligase (E3) to target them for degradation (Lybarger et al, 2005). In addition, recent studies implicated the mK3 polypeptide also with the degradation of TAP and tapasin in the cell (Boname et al, 2004). Calnexin may counteract the association of viral effectors as E19 and mK3 with the PLC and thereby reduce the efficiency of viral interference with the MHC class I antigen presentation pathway. Such a role for the lectin chaperone in maintaining (and possibly protecting) the loading complex appears to be plausible as under conditions of a viral attack the structural integrity and stability of the PLC is likely to be of even greater functional importance as under basal conditions. This is underscored by the high cooperativity among the interactions of the PLC components (Harris et al, 2001; Hughes et al, 1998; Momburg et al, 2002; Sadasivan et al, 1996) to which calnexin would be also likely to contribute. Such a putative role of calnexin in protecting the class I docking sites or conferring additional structural stability on the PLC may also provide an explanation why TAP comprises tapasin-docking sites in both subunits, although the presence of solely one N-domain (in the 2-1ΔN variant) appears to be completely sufficient for optimal peptide loading in all assays that were performed so far.

6.3 The N-region of TAP2 is essential for the structural and functional integrity of the peptide loading complex

One of the crucial findings presented in this thesis is that T2-transfectants expressing variant 1-2ΔN are characterized by a significant thermolability of intracellular MHC class I molecules when compared to the corresponding cell lines expressing 2-1ΔN or wild-type TAP (Fig. 14 and Fig. 15). However, the observation that the two truncated transporters show comparable binding of tapasin and MHC class I (Fig. 16) indicates that the low class I-stability in the cell line T2(1-2ΔN) is not due to a diminished interaction between 1-2ΔN and tapasin. Instead, in view of the well-known correlation between the thermostability of MHC class I molecules and the affinity of their peptide cargo (Williams et al, 2002), it is reasonable to assume that in the presence of variant 1-2ΔN suboptimal ligands are loaded onto class I, which fail to confer high stability on the resulting complex. This is consistent with previous studies that reported a significantly reduced stability of MHC class I molecules in human cell lines expressing tapasin variants, which lack TAP-association (Tan et al, 2002; Williams et al, 2002). As these mutant tapasin molecules fail to recruit the transporter into the PLC, it was concluded that the incorporation of TAP into the loading complex is essential for an optimal quality control of peptide loading. However, by which mechanism TAP improves the stability of class I remained elusive. One promising scenario was that the “bridging function” of tapasin, which allows for a close structural proximity of TAP and MHC class I molecules within the PLC would increase the local peptide concentration. As a direct consequence, the diversity of available ligands for class I was postulated to become expanded, which in turn could lead to the acquisition of more optimal peptide cargo. However, as 1-2ΔN displays normal interaction with MHC class I (Fig. 7B and Fig. 16) the results

described in this thesis demonstrate for the first time that the “bridging function” of tapasin is clearly not sufficient for the formation of stable MHC class I-peptide complexes. Instead, the experiments depicted in Fig. 16 show that the thermolability of MHC class I in the cell line T2(1-2ΔN) (Fig. 14 and Fig. 15) correlates with a drastically reduced amount of accessory chaperones in the 1-2ΔN-associated loading complex (Fig. 16). Nothing comparable is observed in the cell line T2(2-1ΔN) (Fig. 16). Quantitative evaluation of the corresponding immunoblots revealed that the amounts of tapasin and ERp57 co-isolated with 2-1ΔN correspond to approx. 50% of those co-isolated with wild-type TAP. In contrast, the levels of calreticulin and MHC class I associated with 2-1ΔN and TAPwt are roughly identical. This indicates that the transporter variant 2-1ΔN forms a normal class I-loading site via TAP2, but lacks the tapasin/ERp57-interaction in TAP1. However, in the case of variant 1-2ΔN, the signals for the co-isolated chaperones ERp57, calreticulin and calnexin were only barely detectable. Thus, despite the normal association of the TAP1 subunit with tapasin and MHC class I in the TAP variant 1-2ΔN, this truncated transporter seems to be highly defective in the stable recruitment of chaperones. Nevertheless, neither the presence of ERp57 nor that of calreticulin in the PLC was reported to be essential for the process of peptide-loading onto MHC class I (Gao et al, 2002; Howarth et al, 2004). Consistent with this, the kinetics for the release of MHC class I from the PLC were found to be identical among the cell lines T2(TAPwt), T2(1-2ΔN) and T2(2-1ΔN). However, several recent studies point towards a critical role for ERp57 and calreticulin in the quality control of antigen presentation (Dick et al, 2002; Gao et al, 2002). In this context it was speculated that the optimization of peptide cargo for MHC class I within the PLC is a multifactorial process where tapasin in cooperation with the accessory chaperones improves the efficient loading with those ligands that

confer high conformational stability. A recent working model postulates that tapasin optimizes peptides for MHC class I by deforming the peptide-binding pocket in a way that some stabilizing contacts to the peptide cannot be formed (Wright et al, 2004). Under these conditions, which broadly disfavour peptide-binding solely optimal ligands (those that comprise optimal anchor residues) would be expected to resist their dissociation from class I. However, the exact function of ERp57 and calreticulin in the loading complex is currently not known. One may speculate that both contribute to a further deformation of the peptide binding pocket in the class I molecule and thereby assist the tapasin-mediated peptide editing function. Additionally, it is also possible that ERp57, which possesses cysteine protease activity (Urade et al, 1992), acts as a peptide trimming enzyme within the PLC, thereby directly shaping the peptide repertoire loaded onto MHC class I molecules. Furthermore, since calreticulin was shown to bind peptides translocated by TAP (Spee et al, 1997) this lectin chaperone could protect optimal peptide ligands and help to efficiently deliver them onto peptide-receptive class I. Moreover, the results presented in this thesis show for the first time that in addition to ERp57 and calreticulin, also calnexin is a regular component of the mature human PLC (Fig. 17A and Fig. 17D). Although the data regarding the MHC class I stability in CEM cells (Fig. 18C) argues against a general role for calnexin in the quality control of peptide loading it cannot be excluded at present that this lectin chaperone may contribute to the stability of peptide-MHC class I complexes in the case of some MHC class I alleles. Thus, in accordance with the view that the accessory chaperones in the PLC significantly contribute to the generation of highly stable MHC class I-peptide complexes (Dick et al, 2002; Gao et al, 2002), the results shown in Fig. 14 and Fig. 15 suggest that the PLCs formed by 1-2ΔN have lost their quality control function in MHC class I loading by a failure in the chaperone-assisted peptide editing.

Interestingly, the experiments on the TAP variants 2-1ΔN and 1-2ΔN also indicate for the first time that the N-terminal tapasin-docking sites of TAP1 and TAP2 have different requirements for the stable binding of ERp57, calreticulin and calnexin (Fig. 16). In particular, the results shown in Fig. 14 and Fig. 15 point towards a key role for the N-domain in the TAP2 subunit in the proper assembly of the TAP-associated PLC.

As noted before, calnexin was proposed by others to be a component of a TAP/tapasin-precursor-complex that is formed preceding the entry of calreticulin and MHC class I into the PLC (Fig. 4) (Diedrich et al, 2001). As the co-isolation of this chaperone with 1-2ΔN was (besides ERp57 and calreticulin) also dramatically reduced when compared to TAPwt, the impeded formation of a proper loading complex by 1-2ΔN could be due to problems that appear early in the biosynthetic assembly of TAP/tapasin-complexes. Surprisingly, experiments on the TAP single chains and the tandem transporters indicated that depending on its assembly state, TAP1 uses different subregions of its TMD for the interaction with tapasin (Fig. 11A and Fig. 12C). That comparable amounts of tapasin and MHC class I were co-isolated with 1ΔN and wild-type TAP1 (Fig. 12C) suggests further that the core TMD acts as the sole tapasin-docking site in single expressed TAP1 chains. Thus, in view of the findings on 1ΔN, TD1/2ΔN and TD1ΔN/2ΔN (Fig. 11A and 12C), it is reasonable to speculate that TAP subunit pairing is required for the displacement of tapasin from the core TMD to the N-domain of TAP1. Interestingly, a recent study on the biosynthetic assembly of the TAP subunits provides evidence that heterodimerization of TAP occurs in a process where pre-existing (fully synthesized and folded) TAP1 chains associate with newly synthesized TAP2 chains (K. Keusekotten, R. M. Leonhardt, S. Ehses and M. R. Knittler, *manuscript in preparation*). Thus, it is tempting to speculate that the rearrangement of tapasin

within the TMD of TAP1 is directly induced by the entry of the nascent TAP2 chain during the heterodimeric assembly of the transporter. In view of this scenario, the N-domain in TAP2 might have a critical role in stabilizing the interaction with accessory chaperones during the displacement of the tapasin-associated loading complex from the core TMD to the N-domain in TAP1. Consequently, its absence may result in an irreversible loss of accessory chaperones from the PLC or in the persistent failure to stabilize TAP1-associated chaperones during the following loading cycles.

Nevertheless, the question arises, which function the (additional) tapasin-docking-site in the core TMD of TAP1 serves for, as it is apparently not used for the formation of a loading complex in the assembled transporter (Fig. 11A). The answer may lie in the complex network of interactions that are required for the stable high-level expression of the transporter. In particular, several groups could recently show that in contrast to TAP1, TAP2 chains have a reduced *in vivo*-stability when expressed in isolation (Antoniou et al, 2002b; K. Keusekotten, R. M. Leonhardt, S. Ehses and M. R. Knittler, *manuscript in preparation*). Thus, it was proposed that TAP1 is required for the stable expression of TAP2 and the assembled transporter molecule (Furukawa et al, 1999). This is in keeping with observations that the expression level of TAP1 seems to control the amount of stable and functional heterodimeric TAP in the ER (Herzer et al, 2003; Zhu et al, 1999). TAP1 expression in turn depends on its ability to interact with tapasin (Garbi et al, 2000; Tan et al, 2002). This becomes evident by a dramatic decrease in the steady state levels of TAP1 (up to 100-fold) in cells that are tapasin-deficient or express tapasin mutants, which fail to associate with TAP (Garbi et al, 2003; Tan et al, 2002). Interestingly, the steady state expression level of the truncated TAP1 chain 1ΔN is not affected at all when compared to wild-type TAP1, irrespective whether it is expressed as a single subunit (Fig. 12A) or in conjunction with the corresponding TAP2 chain (Fig. 5B). This suggests that tapasin-mediated

stabilization of TAP1 does not depend on the ability of this chaperone to interact with the N-domain of the chain. Consequently, the stabilization of this subunit is likely to occur via tapasin-binding to the core TMD of TAP1 and therefore presumably takes place before and/or during the biosynthetic assembly of the transporter molecule.

6.4 Functional expression of TAP lacking both N-regions can be achieved by head-to-tail fusion of the two chains linked to one another the connector region of MDR1b

Repeated transfection experiments failed to generate T2 cell lines stably co-expressing the TAP chain variants 1ΔN and 2ΔN. That Koch and colleges observed nothing comparable in baculovirus-infected insect cells (Koch et al, 2004) is most likely due to the fact that the authors used a transient over-expression system for their studies. Nevertheless, the experiments shown in Fig. 9, Fig. 10 and Fig. 11 demonstrate that the “co-expression problem” of 1ΔN and 2ΔN in T2 cells can be solved by a head-to-tail fusion of both truncated subunits linked by the 61 amino acid-long connector region of murine MDR1b (Gros et al, 1986). The peptide transport activity of TD1/2ΔN and TD1ΔN/2ΔN (Fig. 10C, Fig. 11B and Fig. 11C) indicates that the fused TAP subunits are able to form the same functional interfaces as they do in heterodimeric wild-type TAP. This strongly supports the hypothesis that the structural organization and pairing of the TMDs and NBDs is highly conserved between “tandem ABC-transporters” and “two chain ABC-transporters”. The connector region of MDR1b and other tandem ABC-transporters shares no detectable sequence homology to the N-domains of TAP and is known to act as a flexible linker that allows proper folding, pairing and coordinated interaction of the two halves of tandem transporters (Hrycyna et al, 1998). Mutations in the connector

region of MDR1 can lead to protein instability, misfolding and abrogation of transport activity (Hrycyna et al, 1998). Thus, although clearly not essential for the expression of single TAP chains (Fig. 12A) or the formation of a functional translocation pore in the heterodimeric transporter (Fig. 6B), the N-domains of TAP1 and TAP2 may be part of a structural unit in TAP that analogous to the connector region is critical for the efficiency of transporter biogenesis by reducing the risk of misassembly and as a consequence proteolytic degradation or accumulation of cell-toxic aggregates. This view is strongly supported by the observation that complex assembly between 1ΔN and 2ΔN is drastically impaired (Fig. 9A). Interestingly, a very recent study reported the construction of two equally functional tandem variants of the homodimeric “half size” ABC transporter ABCG2 (Bhatia et al, 2005), which has been implicated with the acquisition of multidrug resistance in cancer cells (Lockhart et al, 2003). Analogous to the tandem transporters described in this thesis, both variants were generated by head-to-tail fusion of two identical ABCG2 subunits. In one of the two variants the ABCG2 subunits were directly fused to one another whereas in the other variant the connector region of human MDR1 was used as a linker to join both chains. As either variant was shown to be clearly active (Bhatia et al, 2005) it seems that the presence of a connector region in tandem transporters is at least not essential for proper function in general. However, it is noted that ABCG2 has a highly uncommon genetic structure when compared to other ABC transporters (including TAP) as here an N-terminal NBD is followed by a C-terminal TMD (Doyle et al, 1998). Consequently, the domain organization in the ABCG2 head-to-tail fusion gene follows the order *H_2N*-[NBD-TMD]-[NBD-TMD]-*COOH* whereas the order in most tandem transporters (including TD1/2ΔN and TD1ΔN/2ΔN) is *H_2N*-[TMD-NBD]-[TMD-NBD]-*COOH*. Thus, the situation that the connector region in the ABCG2 fusion protein is dispensable for proper folding of the transporter and its substrate

translocation activity (Bhatia et al, 2005) may represent a special case that can be applied for only a few tandem transporters rather than to reflect a fundamental feature of tandem transporters in general. In this context it is interesting to note that indeed some fungal tandem ABC transporters like Pdr5p share the same unusual domain structure as the constructed ABCG2 fusion protein (Bissinger et al, 1994). Thus, one may speculate that this specific class of drug exporters does not require a connector region for folding and substrate transport. Nevertheless, a head-to-tail fusion construct of TAP with N-terminal TAP2 immediately followed by TAP1 without any connector region (*H_2N*-TAP2-TAP1-*COOH*) is only expressed at hardly detectable levels in T2 cells and has no apparent transport activity (J. C. Howard, *personal communication*), indicating (albeit not proving) that the construction of a functional TAP tandem transporter indeed requires the presence of a connector region for proper assembly of the two homologous halves as observed for the related MDR1.

6.5 Proprotein convertases rescue suboptimally loaded MHC class I molecules in late secretory compartments

Characterization of the intracellular MHC class I presentation pathway in cells expressing variant 1-2ΔN (Fig. 15A and Fig. 15C) showed that in a situation where the quality control in the loading complex fails stabilization of ER-exported MHC class I complexes can occur during intracellular transport to the plasma membrane. Very similar observations were also described for tapasin-deficient cells (Williams, et al, 2002). Thus, the underlying cellular rescue mechanism does not appear to be restricted to a situation of malfunctioning TAP, but in contrast is likely to become generally applied by the cell under circumstances where (for whatever reason) unstable MHC class I molecules leave the ER and traffic to the plasma membrane.

Yet, it is unknown whether this process is advantageous for the cellular immune response or rather represents an Achilles heel of the host's ability to efficiently strike against intruding microbes or cancerous cells. As tumours were reported to frequently down-regulate the expression of tapasin (Delp et al, 2000; Giorda et al, 2003; Lou et al, 2005; Raffaghello et al, 2005; Seliger et al, 2003) malignant cells may exploit this cellular rescue pathway to ensure sufficient levels of "self peptide"-presenting MHC class I molecules at the cell surface for evasion of NK cell-mediated killing (Moretta et al, 1996). Conversely, as some viruses interfere with the quality control of peptide loading in the PLC (Park et al, 2004) this pathway could also contribute to a rescue in the class I-mediated presentation of viral peptides and consequently to an enhanced clearance of infected cells (see below). Most interestingly, the studies depicted in Fig. 21 provide for the first time evidence that post-ER stabilization of MHC class I in the cell line T2(1-2ΔN) depends on proprotein convertase activity, but not on endocytic recycling and/or reloading of MHC class I by endosomal processing (Fig. 19). Consistent with this idea the stability of ER-exported MHC class I molecules appears to improve as these migrate along the exocytic route (Fig. 15C). Thus, it is tempting to speculate that the observed phenomenon reflects the exchange by MHC class I of suboptimal (weakly binding) peptide ligands that had been loaded in the ER for high affinity peptides in the TGN. These are presumably derived from prodomains liberated by the proteolytical activity of furin-like convertases (Fig. 24). Several lines of evidence support such a model. First, it is known that less stably associated peptide ligands dissociate from MHC class I at acidic pH-conditions corresponding to the *trans*-Golgi network (determined to be around 5.0) (Chefalo et al, 2003; Stryhn et al, 1996). Furthermore, at an acidic pH of around 5.0 MHC class I molecules are peptide-receptive and can perform efficient peptide exchange (Chefalo et al, 2003; Gromme et al, 1999). Second, several

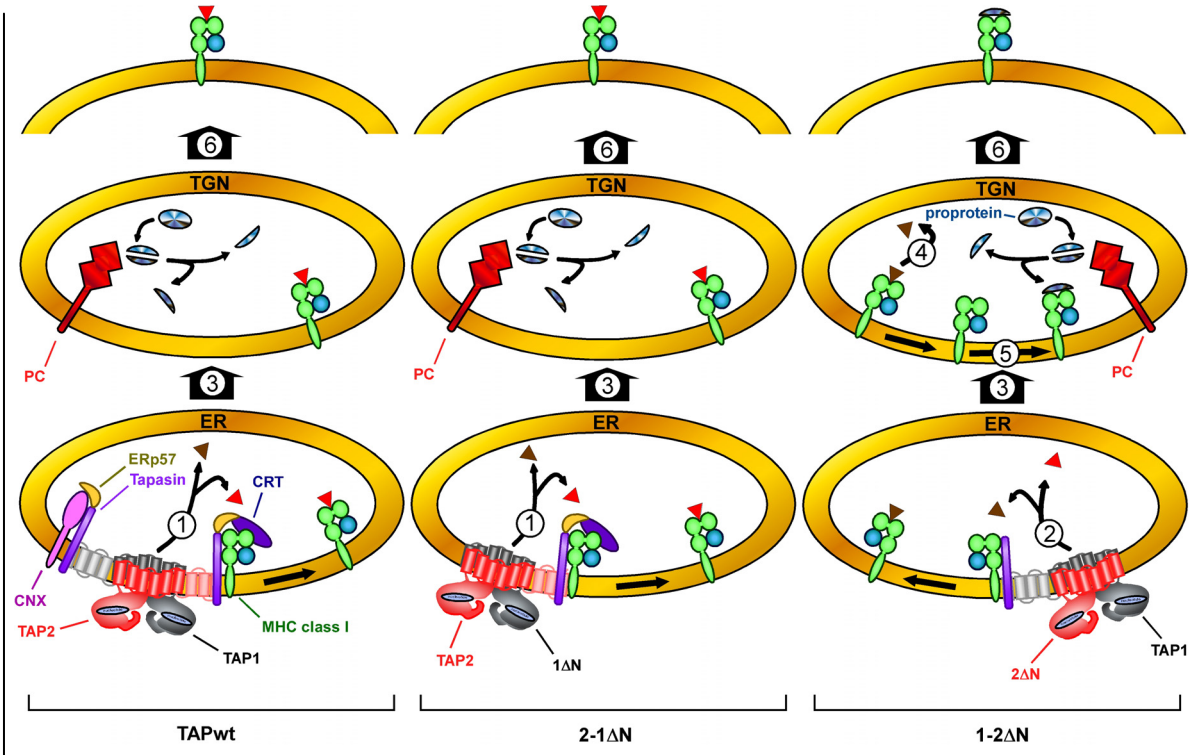

Fig. 24 **Current working-model for proprotein convertase dependent rescue of MHC class I molecules in T2(1-2ΔN) cells**
In the wild-type loading complex the central TAP molecule forms a precursor complex (comprising tapasin, ERp57 and calnexin (CNX)) via one N-domain and a peptide-loading complex (comprising tapasin, ERp57, calreticulin (CRT) and MHC class I) via the opposing N-domain (*left column*). As 2-1ΔN contains solely one of the two tapasin-docking regions present in TAPwt, a normal PLC can only be formed via TAP2 whereas 1ΔN lacks any tapasin interaction in the assembled transporter (*middle column*). In T2(TAPwt) and T2(2-1ΔN) cells the loading of optimal high affinity peptide ligands (red triangle) onto MHC class I is favoured over weakly binding cargo (brown triangle) due to tapasin-mediated peptide-editing (1). Thus the generated peptide-MHC class I complexes are stable when they traverse the TGN and migrate to the cell surface (6). In contrast, 1-2ΔN forms altered PLCs that fail to stably recruit accessory chaperones (*right column*). Consequently, the peptide-editing function of the PLC is disturbed (2), which leads to the transfer of low-affinity peptides (brown triangles) onto MHC class I. The resulting unstable class I molecules become exported from the ER (3) at a normal rate, but loose their suboptimal cargo in the acidic milieu of the TGN (4). Proprotein convertases (PC) cleave proproteins along the secretory route and thereby contribute to the generation of peptides. Although this pathway is active in all T2 transfectants, empty MHC class I molecules largely appear solely in the TGN of T2(1-2ΔN) cells. These are rescued by the binding new PC-dependent ligands (5). As the acidic conditions in the late secretory compartments confer high stringency on the MHC class I-peptide interaction, complexes that can be formed under these circumstances are highly stable. Following the peptide-exchange in the TGN of T2(1-2ΔN) MHC class I becomes exported (6) and migrates to the plasma membrane.

members of the proprotein convertase family, including furin, PACE4, PC5 and PC7 reside in the *trans*-Golgi network or in post-TGN vesicles (Fig. 20C; Seidah et al, 1998; Wouters et al, 1998), where they process a plethora of endogenous ligands including matrix metalloproteinases, integrins, growth factors, receptors, peptide-hormones and neurotransmitters (Khatib et al, 2002). Following the initial cut, the

resulting peptides can be further trimmed by carboxypeptidases (Reznik et al, 2001). Of these at least carboxypeptidase D is ubiquitously expressed and localized to the TGN (Reznik et al, 2001). This indicates that in the compartments of the late secretory route (in most cell types) a whole battery of proteases is capable of generating and processing putative class I ligands. Most importantly, the finding that the MHC class I surface levels in T2(1-2ΔN), but not in T2(TAPwt) or T2(2-1ΔN) cells are sensitive to the highly specific proprotein convertase inhibitor hexa-D-arginine (Fig. 21) provides direct evidence for the involvement of PCs in the rescue of unstable MHC class I molecules. This is in line with several recent reports, which demonstrate that proprotein convertases can contribute peptides to the repertoire of MHC class I-presented epitopes (Gil-Torregrosa et al, 1998; Gil-Torregrosa et al, 2000; Lu et al, 2001; Zhang et al, 2001). However, it is noted that all studies cited above focus on the presentation of individual peptide antigens liberated from chimeric constructs that are normally not expressed in the cell. Thus, the contribution of PCs to the peptide pool presented by MHC class I was in these studies determined under somewhat artificial conditions. In contrast, this thesis describes for the first time the influence of proprotein convertases on the bulk population of class I ligands derived from naturally occurring intracellular proteins. It should additionally be mentioned that MHC class I-presented epitopes liberated from PC-processed viral polypeptides have been described (Johnstone et al, 2004), although it is not yet clear whether proprotein convertases are essential for the generation of the respective peptides. Additionally, the idea that suboptimally loaded MHC class I molecules exchange their peptide cargo in the TGN is further supported by the time-course at which MHC class I molecules acquire stability in T2(1-2ΔN) cells. In particular, the stability of a pulse-labeled post-ER class I population in T2(1-2ΔN) cells continues to improve for clearly more than 3 hours (Fig. 15C). This indicates that a significant

fraction of the respective molecules has already reached the plasma membrane or has at least entered the late compartments of the secretory route (for comparison: the $T_{1/2}$ for MHC class I surface recovery was determined to be approx. 5 hours (Fig. 8B)). Taken together a model can be postulated, in which the TGN serves as a post-ER "checkpoint" of MHC class I processing that rescues unstable MHC class I complexes, which otherwise would have a reduced chance to survive on the cell surface (Fig. 24). This pathway would maintain high (or depending on the allele at least moderate) MHC class I surface levels even under conditions of a malfunctioning quality control of peptide loading. The respective epitopes would derive from secretory proteins and due to a more limited number of substrates (and perhaps proteases) involved, the overall repertoire of presented peptides would be expected to be somewhat narrowed when compared to a cell where the conventional class I antigen presentation pathway works properly. Nevertheless, since MHC class I-bound peptides are much shorter in length than the propeptides liberated by PCs, efficient MHC class I loading would require further processing by other proteolytic enzymes. In particular, proprotein convertase-generated peptides for HLA-B5 are predicted to depend at least on C-terminal trimming as the furin-like convertases cleave after paired basic amino acids whereas HLA-B5 strongly prefers a hydrophobic C-terminal anchor residue (Falk et al, 1995). Although not excluding other possibilities, recent studies suggest that carboxypeptidases serve for that function (Gil-Torregrosa et al, 1998; Lu et al, 2001). Nevertheless, the question arises why peptides generated in the TGN by proprotein convertases can stabilize HLA-B5 in T2(1-2ΔN) cells whereas the ligands available in the ER obviously cannot. It must be considered unlikely that the peptide pool to which PC-activity contributes has in general a higher affinity for MHC class I as the peptides generated by the proteasome. Rather it is plausible to assume that the acidic environment in the TGN

confers high stringency on MHC class I loading and allows only optimal peptide ligands to bind (and stay associated) (Fig. 24).

It is interesting to note that a plethora of viral proteins is processed by members of the PC-family, among them gp160 of HIV (Decroly et al, 1997), F-protein of RSV (Gonzalez-Reyes et al, 2001), P22 of HBV (Messageot et al, 2003) and gpUL55 of HCMV. Furthermore, under conditions of an acute infection viral proteins may become highly abundant in the secretory compartments. Thus, as evasion strategies of some viruses target tapasin-mediated peptide optimization mechanisms and thereby cause the generation of instable MHC class I molecules (Park et al, 2004), the proprotein convertase-dependent rescue of MHC class I complexes could force the surface presentation of viral peptides. Hence, the alternative pathway of MHC class I loading in the TGN may be part of a cellular counter strategy that undermines viral efforts to block the antigen presentation machinery.

Nevertheless, as on the one hand proprotein convertases add peptides to the MHC class I-pathway under certain conditions (Fig. 21 and Fig. 24), they cleavage-activate viral fusion proteins on the other hand and thereby make an essential contribution to the infectivity of the virus (Jean et al, 2000; Kibler et al, 2004). Thus, positive aspects of proprotein convertase expression for the cellular immune response are likely to be counteracted by a high rate of viral replication. Conversely, a downregulation of PCs may result in an overall reduction of infectious viral particles, but may also dampen efficient antigen presentation and thereby limit the chance to ultimately eliminate infected cells. Seven highly related members of the proprotein convertase family have been identified so far (Seidah et al, 1998). Despite some individual characteristics they all share clearly overlapping cleavage specificities and substrates (Basak et al, 2001; Kibler et al, 2004; Vollenweider et al, 1996). The extensive downregulation of at least furin and PACE4 in response to interferons (Fig. 22) is

likely to represent a novel mechanism of innate cell-autonomous immunity that limits the rate of viral spread during an acute infection (see above). It is yet unclear at the molecular level how the transcription of the respective convertases is switched off upon interferon treatment. However, the furin promotor was shown to contain multiple binding sites for the transcription factor SP1 (Ayoubi et al, 1994), which is involved in the interferon-γ-induced transcriptional repression of the lipoprotein lipase gene in macrophages (Hughes et al, 2002). In addition, also the PACE4 promotor was reported to comprise binding sites for SP1 (Tsuji et al, 1997). Therefore, SP1 is one attractive candidate for mediating the down-regulation of proprotein convertases in response to antiviral cytokines. However, that PC7 expression clearly persists upon interferon treatment suggests that this protease may continue to supply MHC class I with ligands even under conditions of a viral infection. Possibly, the presence of this single convertase is not sufficient to allow for an optimal replication of most viruses. As several viral proteins require the cleavage at multiple sites for proper maturation (Gonzalez-Reyes et al, 2001; Messageot et al, 2003) and some of these may not be favoured by PC7, the presence of only one (or only a few) different convertases in the TGN may ensure an efficient generation of peptides without allowing for a high viral replication rate. Nevertheless, the results shown in Fig. 22 do not rule out a possible role for furin and PACE4 in the presentation of viral antigens in the context of an infection, as several viruses have been reported to block the interferon signalling pathway, among them HCMV (Miller et al, 1998; Miller et al, 1999), hepatitis C virus (HCV) (Duong et al, 2004) and varicella-zoster virus (VZV) (Abendroth et al, 2000). One possible reason why viruses tend to inhibit interferon-signalling could be that they require specific proprotein convertases as furin or PACE4 (or they require higher levels of PCs in general) for efficient replication and

spread. The results presented in this thesis suggest that they may pay a high price for the forced (re-)onset of proprotein convertase expression.

7. Summary

The transporter associated with antigen processing (TAP) translocates antigenic peptides into the ER for binding onto MHC class I molecules. Tapasin organizes a peptide-loading complex (PLC) by recruiting MHC class I and accessory chaperones to the N-terminal regions (N-domains) of the TAP subunits TAP1 and TAP2. To investigate the function of the tapasin-docking sites of TAP in MHC class I processing, N-terminally truncated variants of TAP1 and TAP2 were expressed in combination with wild-type chains, as fusion proteins or as single subunits. The results indicate that TAP interacts at steady state with MHC class I solely via one N-domain whereas the opposing tapasin-docking site concomitantly associates with calnexin. Strikingly, TAP variants lacking the N-domain in TAP2, but not in TAP1, build PLCs that fail to generate stable MHC class I-peptide complexes. This correlates with a substantially reduced recruitment of accessory chaperones into the PLC demonstrating their important role in the quality control of MHC class I loading. However, stable surface expression of MHC class I is rescued in compartments of the late secretory route by a mechanism that depends on the proteolytical activity of the proprotein convertase family. Finally, it is shown that proprotein convertase expression is differentially modulated in response to interferons indicating that individual members of this protease family may make distinct contributions to an antiviral immune response.

8. Zusammenfassung

Der Peptidtransporter TAP überführt Peptidantigene aus dem Cytosol in das endoplasmatische Retikulum (ER). Diese werden anschließend auf MHC Klasse I-Moleküle geladen. Tapasin spielt eine zentrale Rolle bei der Organisation des sogenannten Peptid-Ladekomplexes, welcher neben TAP und MHC Klasse I-Molekülen auch akzessorische Chaperone, wie Calreticulin und ERp57 enthält. Beide Untereinheiten des heterodimeren Transporters, TAP1 und TAP2, interagieren mit Tapasin über ihren N-Terminus, die sogenannte N-Domäne. Um die Funktion der Tapasin-Bindestellen in den beiden TAP-Ketten zu ermitteln, wurden Deletionsmutanten der TAP-Untereinheiten erzeugt, in denen jeweils die N-Domänen entfernt wurden. Diese wurden dann entweder gemeinsam mit der komplementären wildtypischen Untereinheit, als Fusionsprotein oder als einzelne Ketten in einem humanen TAP-negativen Zellsystem exprimiert. Es konnte gezeigt werden, daß der Transporter zu jedem gegebenen Zeitpunkt in der Zelle lediglich über eine seiner beiden Tapasin-Bindestellen mit MHC Klasse I-Molekülen assoziiert ist. Dagegen interagiert die gegenüberliegende N-Domäne mit dem ER-Chaperon Calnexin. Weiterhin ergab sich, daß das Vorhandensein der N-Domäne in TAP2, nicht aber der korrespondierenden N-Domäne in TAP1 für die Beladung von MHC Klasse I-Molekülen mit stabil bindenden, hochaffinen Peptid-Liganden essentiell ist. Eine TAP-Deletionsvariante, in welcher der N-Terminus in TAP2 entfernt worden war, bildete zwar Peptid-Ladekomplexe aus, allerdings zeigten diese eine deutlich schwächere Interaktion mit akzessorischen Chaperonen. Im Einklang mit anderen Studien verdeutlicht dies die herausgehobene Rolle dieser Chaperone für die Qualitätskontrolle der Peptidbeladung von MHC Klasse I-Molekülen. Interessanterweise konnte jedoch gezeigt werden, daß die Stabilität suboptimal beladener MHC Klasse I-Moleküle nach Verlassen des ERs in späten sekretorischen

Kompartimenten wiederhergestellt werden kann. Dieses Phänomen ist abhängig von der proteolytischen Aktivität sogenannter Proprotein-Konvertasen, die sich im *trans*-Golgi Netz befinden und dort an der Prozessierung einer Vielzahl zellulärer Polypeptide beteiligt sind. Daher legen die erzielten Ergebnisse nahe, daß die Zunahme der Stabilität mangelhaft beladener MHC Klasse I-Moleküle durch den Austausch von Peptid-Liganden erfolgt. Darüber hinaus konnte gezeigt werden, daß die Transkription der verschiedenen Proprotein-Konvertasen unterschiedlich durch Interferone moduliert wird, was daraufhin deutet, daß unterschiedliche Mitglieder dieser Protease-Familie unterschiedliche Aufgaben während einer antiviralen Immunantwort übernehmen.

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