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Critical Role for the Tapasin-Docking Site of TAP2 in the Functional Integrity of the MHC Class I-Peptide-Loading Complex

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The transporter associated with Ag processing (TAP) transports cytosolic peptides, predominantly generated by the proteasome into the endoplasmic reticulum (ER) (1). There, they are loaded onto newly synthesized MHC class I (MHC I) that transiently associates with TAP and accessory chaperones to form the MHC I peptide-loading complex (PLC) (1).

Tapasin, a type I transmembrane glycoprotein, is believed to play a key role in the formation of the PLC because it binds to MHC I molecules (2) as well as to the transmembrane domains (TMDs) of the TAP subunits, TAP1 and TAP2 (3). In the absence of tapasin the stability of MHC I-peptide complexes is drastically reduced (4) and the repertoire of surface-presented peptide Ags is altered (5), suggesting that tapasin acts as an editor that selects peptides for stable binding onto MHC I (6). Importantly, loading of MHC I with peptide Ags conferring high stability requires the tapasin-mediated introduction of TAP into the PLC, whereas the mere interaction of MHC I with tapasin is not sufficient (7). However, whether the close proximity between MHC I and TAP in the PLC, as such, favors tapasin-mediated quality control of MHC I loading by high local peptide concentration or whether the interaction of tapasin with TAP1 and/or TAP2 has a more specific function in the process of MHC I loading is not known.

A current study showed that coexpression of truncated TAP chains lacking the N-terminal 4 and 3 transmembrane helices in TAP1 and TAP2 results in an active peptide transporter that is deficient in tapasin association (8). Because the TMDs of TAP1 and TAP2 were shown to bind tapasin independently from each other (3), it was reasoned that the interaction of tapasin with TAP occurs exclusively via the N-terminal regions (N domains) of both TAP subunits.

To investigate whether and to what extent the N domains of TAP are required for intracellular processing and surface expression of MHC I, we stably expressed truncated TAP subunits in combination with corresponding wild-type TAP chains, as head-to-tail fusion proteins or as single subunits in the human TAP-negative cell line T2. We show that the N domains of both TAP subunits operate as separate tapasin binding sites in the PLC. At any given time only one of two N domains appears to be actively involved in the tapasin-mediated interaction with newly synthesized MHC I. However, neither N domain is required for ER export and surface presentation of MHC I. Moreover, depending on the assembly state of TAP1, different subregions of its TMD are used for tapasin-binding. Heterodimeric assembly of the TAP subunits is accompanied by a displacement of tapasin from the coreTMD to the N domain in TAP1, whereas TAP2 interacts with tapasin exclusively via the N domain.

An important finding of our work is that TAP variants lacking the N domain in TAP2, but not in TAP1, form PLCs that are disturbed in the physical interaction with calreticulin (Crt), calnexin (Cnx), and ER60 and the quality control of MHC I loading. This suggests that TAP1 and TAP2 differ in the structural requirements for stable recruitment of accessory chaperones and that the tapasin-docking site of TAP2 plays a pivotal role in the functional integrity of the PLC. In a situation where the quality control function of the PLC fails, a post-ER mechanism rescues stable peptide-loading and surface expression of MHC I. Most interestingly, our findings suggest that this depends on the catalytic activity of proprotein convertases (pPCs) that reside in compartments of the late exocytic route (9) and contribute to the proteolytic generation of CTL-epitopes (10).

The results of this report extend our knowledge on the function of the PLC and contribute to the understanding of the intracellular
matured of MHC I molecules in the ER and post-ER compartments.

Materials and Methods

Cell lines and cell culture

T2 is a human lymphoblastoid cell line deficient for TAP and expresses HLA-A2 and HLA-B5 (11). Transfectants of T2 expressing rat TAPwt or truncated rat TAPs were cultured in IMDM (Invitrogen Life Technologies) supplemented with 1 mg/ml G418 (PAA Laboratories) (12). Murine TIB-75 hepatocytes (13) were cultured in DMEM (Invitrogen Life Technologies).

Cloning and expression of truncated TAPs

The vector pBLSKII+ (Stratagene) containing rat TAP1Δ cDNA (14) or rat TAP2Δ cDNA (15) was subjected to QuikChange site-directed mutagenesis (Stratagene) using primers 5′-GCGACCGCTGATGCTGCTCCGGACA CAAAGGGCCCTGG-3′ and 5′-CCAGCCGCTCCTTGCTCCCGGAGGCAT CGACGGTGCGCC-3′ or primers 5′-GGCAAGACCCACATCCTGCCGG GAGGAGAATACGAAGGC-3′ and 5′-GCTCTGTATTTCTCCTCTGCCC GGCATGTTGGGTTGTCATTG-3′ to generate 1ΔN (TAP1Δ2-128) and 1ΔN (TAP2Δ2-128) before cloning into pBHAprino (16). Constructs were transferred into T2 cells by using a Bio-Rad gene pulser at 270 V and 500 μF. For cloning of the two tandem TAPs the 3269-bp ScalI/Acc651 fragment from the pBLSKII+ derivative pH2.2ΔN was ligated to either the 4359- or the 3971-bp fragment from a ScalI/BstGI digest of pH2.2ΔN containing TAP1 or 1ΔN. From the resulting plasmids the 7088- or 6675-bp AgeI/BstCI fragment was ligated to the AgeI/BstCI fragment containing the connector region of murine multidrug resistance protein MDR1b (17) (see below). These constructs encoding tandem TAP 1ΔN/2ΔN (TAP1-MDR1b640–701-TAP21ΔN-130) or TD1ΔN/2ΔN (TAP1Δ2-128-MDR1b40–701-TAP2Δ2-130) were cloned into pH2ΔAprino and transfected into T2 cells. The multidrug resistance protein 1b (MDR1b) connector region was cloned by RT-PCR. mRNA was prepared from TIB-75 hepatocytes using RNeasy and Oligotex kits (Qiagen). RT-PCR and subsequent PCR were performed in combination with MDRTAP1-specific primers 5′-CCCCCTACACCTGCTGAGCGAATACTATGCG-3′, 5′-CCTCTAC AATCTGCTGAAATATCTGACCG-3′, and 5′-GGTGTTGCTACCTGTGGAGG-3′. The resulting 403-bp fragment of MDR1b was cleaved with SacI and HindIII and ligated with SacI/Acc651-digested pH2.2ΔN. This vector served as template for a further PCR using the primers 5′-TGTTC CCGGTCATCGTGAGGCTCTTGCGCATTCCAGCAGGAA ATAATGCTTATGGATCCC-3′ and 5′-ATTCGCCGCTGGGACACGCTCA GCAACGCCGAAAAAACCCAGGAGGC-3′ to generate a TAP-MDR1b chimera 17-bp fragment that was ligated with AgeI/BstCI fragments derived from the tandem precursor plasmids (see above). All TAP constructs were fully sequenced.

Antibodies

116/5 and D90 are rabbit antiserum recognizing the C terminus of rat TAP2 (18) or rat TAP1 (15). 3B10.7 is a rat mAb binding the H chains of HLA-A and -B (19). 4E is a β2m-independent mouse mAb that recognizes an epitope common to all assembled HLA-B molecules (20). 0ER60 (21) and Rgp48N (22) are rabbit antisera binding ER60 or tapasin. SPA-600 and -865 are rabbit antiserum recognizing Ctx or Cnx (StressGen Biotechnologies).

Pulse-chase analysis, immunoprecipitation, endoglycosidase H (endo H) assay, Western blotting, and peptide cross-linking

Cells were starved for 2 h in methionine/cysteine-free RPMI 1640. A total of 500 μCi of Pronix (Amersham Biosciences) was added for 30 or 60 min. The chase was initiated by the addition of a 10-fold excess of unlabeled methionine/cysteine. Equal number of cells were lysed in PBS containing 1% digitonin (WAKO) or 1% Triton X-100 (Sigma-Aldrich). Immunoprecipitations were performed from equivalent amounts of precleared lysates by using anti-TAP2 or anti-MHC I Abs. For identification of coisolated MHC I, precipitated complexes were separated by SDS-PAGE. Fluorograms were scanned by a Chromoscan 3 microdensitometer (Joyce-Loeb). Immunoprecipitation, endo H assay, Western blotting, and peptide cross-linking were performed as described earlier (12).

Flow cytometry

Flow cytometry was performed as described previously (12). To investigate the intracellular processing of MHC I molecules, transfectants were incubated for 10 min at 4°C, 30°C, 50°C, and 60°C. Colocalization in the ER and post-ER compartments

maturation of MHC I molecules in the ER and post-ER compartments.

To characterize MHC I processing, primacrine (Sigma-Aldrich) or NH4Cl were applied for 3 or 18 h at a concentration of 250 μM or 20 mM. Hexa-D-arginine (Calbiochem) was added at different concentrations for 1 h at 37°C after removal of surface MHC I by 3 min incubation with 50 mM citrate (pH 3) and subsequent neutralization of pH.

Peptide transport assay

The peptide transport assay was performed as described previously (12). To directly compare the transport activities of TAPwt and truncated TAPs we normalized the results of the transport assays for the expression levels of the different transporters. For quantification of TAP steady-state levels, equivalent number of cells were lysed in 1% Triton X-100. Lysates were serially diluted 2-fold and separated by SDS-PAGE. Western blots were probed for TAP1 and TAP2. Densitometric scanning revealed almost identical expression levels for TAPwt, 1-2ΔN, and 2-1ΔN. In the case of TD1/2ΔN and TD1ΔN/2ΔN, the TAP steady-state expression levels were 10-fold lower compared with that in T2(TAPwt).

Results

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

To investigate the function of the N domains in TAP for MHC I processing, we stably coexpressed rat TAP1, lacking residues 2-139 (1ΔN) or rat TAP2, lacking residues 2-128 (2ΔN) (Fig. 1A) with the corresponding wild-type TAP chain in T2 cells. The respective transporters were named 2-1ΔN and 1-2ΔN. Surprisingly, transporters coexpressing 1ΔN and 2ΔN could not be established, although >100 different cell lines from four independent transfections were analyzed (data not shown). This suggests that stable expression of a heterodimeric TAP variant lacking both N domains is either harmful to the transfected T2 cell or that the transporter is structurally unstable and rapidly degraded. Nevertheless, 1-2ΔN and 2-1ΔN are expressed at levels comparable to TAPwt (Fig. 1B).

Furthermore, studies on peptide binding (Fig. 1C) and transport (Fig. 1D) using radiolabeled model peptides demonstrate efficient transport function for 1-2ΔN and 2-1ΔN.

Based on structural data of ATP-binding cassette transporters (23), we speculated that the N domains of TAP1 and TAP2 point in the heterodimeric transporter to opposite sides of the paired coreTMDs and act as two autonomous tapasin-docking sites for the transient interaction with MHC I. To investigate this, we analyzed the ability of the truncated TAPs to interact with tapasin and MHC I by immunosolation of TAP or MHC I from digitonin-lysed cells (Fig. 2A). Both truncated TAPs retain the ability to form complexes with tapasin and MHC I. However, anti-TAP immunoprecipitations show that tapasin-interaction is reduced in the truncated TAP variants by ~50% when compared with TAPwt. In contrast, the amounts of MHC I coprecipitated with TAP are almost identical for the different transporter variants indicating that MHC I interaction is comparable in TAPwt, 1-2ΔN and 2-1ΔN. In accordance with this, pulse-chase experiments (Fig. 2B) revealed that the half-life of interaction between newly synthesized MHC I and truncated TAP variants is very similar to that observed for the wild-type PLCs (~100 min). Thus, despite reduced tapasin-binding, the PLCs formed by 1-2ΔN and 2-1ΔN show a normal transient interaction with peptide-receptive MHC I. We next compared the intracellular processing (Fig. 2C) and surface expression (Fig. 2D) of MHC I in the different transfectants. Endo H assays with cell extracts from T2(TAPwt), T2(1-2ΔN), and T2(2-1ΔN) (Fig. 2C) revealed that under steady-state conditions ~80% of MHC I is present in post-ER compartments whereas in nontransfected T2 cells >80% of MHC I is retained in the ER. In accordance with this, T2(1-2ΔN), T2(2-1ΔN), and T2(TAPwt) display comparable
surface staining of HLA-B5 (Fig. 2D). Thus, neither the N domain of TAP1 nor the N domain of TAP2 is required for ER-export and surface expression of MHC I. Moreover, our data provide evidence that TAPwt, 1-2ΔN/H9004N, and 2-1ΔN/H9004N do not differ in the transient recruitment of MHC I for peptide loading.

**Head-to-tail-fusion of TAP chains allows stable expression of transporters lacking both N domains**

We next asked whether the construction of a TAP-fusion protein could solve difficulties to stably coexpress 1-2ΔN and 2-1ΔN. Therefore, we used the tandem structure of the P-glycoprotein as template for a head-to-tail fusion of 1ΔN and 2ΔN linked by the connector region of murine MDR1b (17) (Fig. 3A). In addition, a second TAP-fusion construct was made containing TAP1 instead of 1ΔN. The resulting tandem TAPs, TD1ΔN/2ΔN and TD1/2ΔN, which were stably expressed in T2 cells (Fig. 3B) show a subcellular localization comparable to TAPwt (data not shown). For functional characterization we analyzed peptide binding (Fig. 3C) and translocation (Fig. 3D). Both tandem variants show detectable peptide cross-linking, suggesting that they contain functional substrate binding sites. Moreover, the transport activities obtained for TD1/2ΔN and TD1ΔN/2ΔN (Fig. 3D) are roughly within the range of TAPwt when normalized for the expression levels of the different TAP variants. Variant TD1/2ΔN is somewhat less active while variant TD1ΔN/2ΔN is slightly more active than the wildtype transporter. Thus, TAP variants lacking the N domains in both

**FIGURE 1.** Expression and transport function of TAP variants lacking the N domain of TAP1 or TAP2. A, Schematic diagrams of wild-type TAP subunits, 1ΔN and 2ΔN. N domains (ND), coreTMD, transmembrane segments, and NBDs are indicated. Topology prediction of the TMDs was performed with TopPred II (42). The regions corresponding to the tapasin-docking sites (8) are indicated. B, Expression of 1-2ΔN or 2-1ΔN. Cell lysates were analyzed in Western blots probed for TAP1 or TAP2. TAP1 (black) and TAP2 (gray) are shown as pictograms. C, Peptide-binding of TAPwt, 1-2ΔN, and 2-1ΔN. Cell membranes were incubated with radiolabeled and HSAB-conjugated peptide S8 (TVDNKTRYR). After UV-cross-linking and membrane lysis, TAP was immunoprecipitated with anti-TAP2 antiserum. Asterisks indicate unidentified cross-link products. D, Peptide transport activity. Cells were permeabilized and incubated with radiolabeled peptides S5 (TVDNKTRYV) or S8 in the presence of ATP. The transport activity measured for 1-2ΔN and 2-1ΔN is expressed as a percentage of the transport activity (quantified by gamma counting) obtained for TAPwt.
subunits are functional peptide transporters when expressed as head-to-tail fusion constructs.

To analyze the PLCs formed by tandem TAPs, we immunoisolated TAP or MHC I from digitonin-lysed cells and analyzed coisolated polypeptides by Western blot. As with 1–2ΔN (Fig. 2A), the related transporter TD1/2ΔN retains tapasin- and MHC I-binding, whereas TD1ΔN/2ΔN, which lacks both N domains, is completely excluded from the PLC (Fig. 4A). We next investigated whether TD1/2ΔN and TD1ΔN/2ΔN deliver peptides for loading of MHC I. Because the expression levels of the tandem TAPs are lower than the expression levels of TAPwt (Fig. 3B), the cell lines T2(TD1/2ΔN) and T2(TD1ΔN/2ΔN) display only a partial restoration of intracellular processing (Fig. 4B) and surface expression (Fig. 4C) of MHC I. However, despite the differences in the tapasin/MHC I recruitment by the two tandem TAPs, T2(TD1/2ΔN) and T2(TD1ΔN/2ΔN) show very similar steady-state distributions of ER and post-ER MHC I (Fig. 4B) and comparable amounts of surface HLA-B5 (Fig. 4C). This implies that under conditions, in which physical interaction between TAP and tapasin is impaired, newly synthesized MHC 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.

Assembled and nonassembled TAP1 uses different TMD subregions for tapasin binding

The interaction between tapasin and the N domains could depend on heterodimerization of TAP or might reflect an intrinsic property of the two TAP subunits. To address this, we generated T2 cells expressing 1ΔN or 2ΔN as single TAP chains. Fig. 5A shows that both have steady-state expression levels comparable to the corresponding wild-type TAP subunits. To investigate whether the truncated TAP chains retain the ability to associate with the PLC, MHC I and truncated TAP chains were immunoisolated from digitonin lysates and coprecipitated proteins were analyzed by Western blot (Fig. 5B). In contrast to 2ΔN, which is excluded from the PLC, 1ΔN retains the full ability to interact with tapasin and MHC I, indicating that in single expressed TAP1 the coreTMD acts as sole tapasin-docking site. Because indirect immunofluorescence showed identical ER-distribution for the truncated and wild-type TAP subunits (data not shown), we can exclude that the different behavior of 1ΔN and 2ΔN is caused by a distinctive intracellular localization. Thus, our findings suggest 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.
TAP variants 1-2ΔN and 2-1ΔN show different effects on the quality control of Ag presentation

MHC I-loading with peptides conferring high stability requires the tapasin-mediated introduction of TAP into the PLC (7). To investigate the role of the N domains for the quality control of MHC I loading (1), we took advantage of the correlation between the thermostability of MHC I and the affinity of their peptide cargo (6). Therefore, lysates of the different transfectants were incubated for 1 h at 4°C or 37°C before the integrity of MHC I was tested by immunoprecipitation with the HLA-B5-specific mAb 4E and incubation with radiolabeled peptides S5 or S8 in the presence of ATP. The transport activity measured for TD1/2ΔN and TD1ΔN/2ΔN is expressed as a percentage of the transport activity (quantified by gamma counting) obtained for TAPwt after normalization for TAP expression.

2-1ΔN are characterized by high thermostability of intracellular HLA-B5 whereas in nontransfected T2 cells only 20% of intracellular HLA-B5 remains stable at 40°C and ≤10% at temperatures >50°C (Fig. 6B). In line with the results in Fig. 6A, T2(1-2ΔN) shows a remarkable thermostability of intracellular HLA-B5 at temperatures >40°C (40–70% reduction of the immunostaining at 50°C and 60°C, respectively). Most surprisingly, all analyzed transfectants displayed a comparable high thermostability of surface HLA-B5. Accordingly, we observed that in the presence of brefeldin A, which blocks the ER/Golgi transport, the time course in the survival of surface HLA-B5 (≈1/2 = 15 h) is almost identical among T2(1-2ΔN), T2(2-1ΔN), and T2(TAPwt) (Fig. 6C). Taken together, this suggests that in the cell line T2(1-2ΔN) the population of HLA-B5 on the cell surface is physically more stable than the intracellular fraction of HLA-B5.

To investigate whether stabilization of HLA-B5 occurs during the intracellular transport to the cell surface, we performed pulse-chase experiments (Fig. 6D). Cell lysates of different time points were heat-treated at 37°C for 1 h in the presence or absence of MHC I-binding peptides. Thermoreistant MHC I was immunolated with conformation-sensitive mAb 4E and incubated with endo H. The ER-export rate of total HLA-B5 is similar in T2(TAPwt), T2(1-2ΔN), and T2(2-1ΔN) as determined in the presence of stabilizing peptide (Fig. 6D). However, in the absence of external peptide, MHC I from T2(1-2ΔN), but not from T2(TAPwt) or T2(2-1ΔN), shows significant thermostability even after export from the ER (Fig. 6D). Furthermore, comparison of the signals of endo H-resistant MHC I isolated in the presence or in the absence of stabilizing peptides demonstrates that in T2(1-2ΔN) the ER-exported MHC I molecules improve their thermostability during post-ER maturation.
Comparison of MHC class I processing in cells expressing TAP variants 1–2ΔN and 2–1ΔN

Two important questions arise from our findings. First, what is the molecular basis for the defective quality control of MHC I processing in T2(1–2ΔN) and second, what kind of cellular mechanism leads to stabilization of surface MHC I in this cell line?

In addition to tapasin, the chaperones ER60 and Crt are thought to play a critical role in the quality control of MHC I processing (1). Therefore, we compared the complex formation of 1–2ΔN and 2–1ΔN with ER60 and Crt by immunoprecipitation. Additionally, the association with Cnx, which is thought to be a component of a precursor complex that precedes the formation of the PLC (25), was analyzed. Fig. 7A shows comparable precipitation of TAP, tapasin, ER60, and Crt by immunoprecipitation. Furthermore, in accordance with the experiment shown in Fig. 2, we observed comparable co-isolation of MHC I. In support of the model that Crt becomes inserted into the PLC together with MHC I (25), we found identical amounts of this chaperone in the complexes of TAPwt and 2–1ΔN. The amount of tapasin, ER60, and Cnx co-isolated with 2–1ΔN equals half the amount co-isolated with TAPwt, indicating that in 2–1ΔN solely the N domain of TAP2 is involved in the formation of precursor and/or PLCs. The interaction of 1–2ΔN with tapasin and MHC I resemble the properties of complexes formed by 2–1ΔN (Figs. 2 and 7A). However, in contrast to 2–1ΔN, the amount of chaperones coprecipitated with 1–2ΔN is significantly reduced. The amounts of ER60, Cnx, and Crt correspond to 10, 15, and 40% of that found for the complex of TAPwt. Thus, although tapasin and MHC I are efficiently recruited to the N domain of TAP1 in 1–2ΔN, the formation of precursor complexes and PLCs seems to be disturbed in the assembly of chaperones. It is reasonable to assume that this is directly responsible for the defective quality control of MHC I-loading observed in T2(1–2ΔN).

To determine whether the transfectants expressing 1–2ΔN gain stability of surface HLA-B5 by an increased rate of endocytic recycling and reloading of MHC I during endosomal processing, cells were treated with primaquine or NH₄Cl (Fig. 7B). Primaquine is a drug that causes intracellular accumulation of endocytosed proteins including MHC I, whereas NH₄Cl blocks endosomal processing. The different transfectants show almost identical reductions of surface-exposed HLA-B5 in the presence of the lysosomotropic reagents (up to 25% for primaquine). This indicates that the cell lines do not differ in the endocytic recycling and processing of surface MHC I. Stryhn et al. (26) proposed that suboptimally loaded MHC I exchanges low affinity peptide ligands after reaching the late compartments of the exocytic route. Proprotein convertases (pPCs), which cycle between trans-Golgi network (TGN), plasma membrane and endosomes (9) have been reported to be involved in the generation of peptide Ags for MHC I (10, 27). To test whether the high level of surface MHC I expression in cell line T2(1–2ΔN) depends on the activity of pPCs, we performed surface MHC I-recovery experiments in the presence of increasing
FIGURE 6. Thermostability of MHC I molecules. A, Thermostability of MHC I in cell extracts. Left, Lysates of the transfectants were pretreated or not with 10 μM HLA-B5 binding peptide EBV-3C/881-9 (QPRAPIRPI) (43) and incubated for 1 h at 4°C or 37°C. The thermostability of HLA-B5 was analyzed by immunoprecipitation using mAbs 3B10.7 or 4E (left). Precipitates were analyzed in Western blots probed with mAb 3B10.7. The obtained signals were quantified by densitometric scanning and the peak integrals were plotted in arbitrary units (right). B, Thermostability of intracellular and cell surface MHC I. Transfectants were collected in PBS/0.1% NaN₃ and incubated at 4°C, 40°C, 50°C, and 60°C for 10 min. To determine thermostability of surface HLA-B5, cells were directly immunostained with mAb 4E. For the analysis of the thermostability of intracellular HLA-B5, transfectants were permeabilized, fixed, and stained with mAb 4E. Flow cytometry was performed as described in Fig. 2D. C, Survival of MHC I molecules on the cell surface. Transfectants were treated with brefeldin A that inhibits protein export from the ER. After culturing the cells for different time points, MHC I surface staining with mAb 4E was performed. Results of the flow cytometry are presented as percentage of reduction of the mean fluorescence intensities at 1, 2,
concentrations of the pPC-inhibitor hexa-D-arginine (HDA), which inhibits furin and closely related pPCs with high specificity (28). In T2(TAPwt) and T2(2-1N/H9004N) the recovery of surface MHC I was not or only slightly affected. This suggests that in these two cell lines the pPC-pathway plays only a minor role in the generation of MHC I ligands (Fig. 7B). However, T2 cells expressing 1-2N/H9004N show a significant and concentration-dependent reduction (up to 45% at 100 μM HDA) of surface MHC I in the presence of HDA (Fig. 7B), suggesting that a substantial amount of HLA-B5 Ags originate from proteolytic products generated by furin or related pPCs. Thus, it seems that in a situation where quality control in the PLC fails, a post-ER mechanism that depends on pPC-activity can rescue stable surface expression of MHC I.

**Discussion**

Optimization of peptide-loading onto MHC I requires the tapasin-mediated incorporation of TAP into the PLC (7). Recent reports showed that both TAP subunits interact with tapasin (3), and that in assembled transporters the N domains of the TMDs serve as tapasin-docking sites (8). However, the stoichiometry of the TAP/tapasin subcomplex is controversial. The proposed number of tapasin molecules in the PLC ranges from 1 (29) to 4 (30). Thus, it is not clear whether all tapasin-docking sites of TAP are used in the PLC for the MHC I binding at the same time. To investigate the function of the tapasin binding sites in TAP1 and TAP2 for MHC I processing, we stably expressed transporters lacking the N domains in TAP1 (2-1N/H9004N) or TAP2 (1-2N/H9004N) in the cell line T2. Pulse-chase experiments showed almost identical kinetics for the release of MHC I from TAPwt and the truncated TAPs (Fig. 2B). This and the observation that almost equal amounts of radiolabeled MHC I were coisolated with TAPwt, 1-2N/H9004N, or 2-1N/H9004N at any time point of the pulse-chase experiment (Fig. 2B) indicates that during the chase period comparable quantities of newly synthesized radiolabeled MHC I were coisolated with TAPwt, 1-2N, or 2-1N at any time point of the pulse-chase experiment (Fig. 2B) indicates that during the chase period comparable quantities of newly synthesized radiolabeled MHC I passed through the three different PLCs. Consistently, Western blots show that under steady state conditions nearly the same amounts of MHC I bind to the PLCs of wild-type

4, 12, and 15 h compared with the mean fluorescence intensity at 0 h. D, Maturation and thermostability of MHC I. Transfectants were pulse labeled for 30 min and chased for the indicated time points. Cells were lysed in the presence or in the absence of 10 μM peptide EBV-3C/881-9 and incubated for 1 h at 37°C. Immunoprecipitates recognized by mAb 4E were digested with endo H and separated by SDS-PAGE (left). Obtained signals of MHC I H chains (ER and post-ER) were quantified and the bands with the highest intensity were set to 100% (right).
and truncated TAPs, whereas the amount of tapasin coisolated with 1-2ΔN or 2-1ΔN equals half of that associated with TAPwt (Fig. 7A). Thus, although TAPwt has the capability to recruit MHC 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 interaction with newly synthesized MHC I. In view of the observation that the N domains of TAP1 and TAP2 function as separate tapasin-docking sites (Figs. 2A and 7A), it is attractive to speculate that the two TAP subunits in TAPwt normally alternate with each other in the tapasin-dependent recruitment of newly synthesized MHC I.

One of the crucial findings of this work is that T2 transfectants expressing 1-2ΔN display significant thermolability of intracellular MHC I when compared with cell lines expressing 2-1ΔN or TAPwt (Fig. 6). In view of the well-known correlation between the thermostability of MHC I and the affinity of their ligands (6), it is reasonable to assume that in the presence of 1-2ΔN MHC I is loaded with suboptimal peptide cargo. The observation that the truncated TAPs, 1-2ΔN and 2-1ΔN, show comparable binding of tapasin and MHC I (Fig. 7A) excludes that the low MHC I stability in cell line T2(1-2ΔN) is due to a diminished interaction between 1-2ΔN and tapasin, thus, the “bridging function” of tapasin, which allows the close proximity of TAP and MHC I, is apparently not sufficient for the formation of stable MHC I-peptide complexes. Most interestingly, the thermostability of MHC I in T2(1-2ΔN) correlates with a drastically reduced amount of chaperones in the 1-2ΔN-associated PLC (Fig. 7A). Quantitative evaluation revealed that the amount of chaperones coisolated with 2-1ΔN corresponds to half the amount of auxiliary molecules associated with TAPwt whereas in the case of 1-2ΔN, the signals for the coisolated chaperones are only barely detectable. Thus, despite the normal association with tapasin and MHC I, 1-2ΔN seems to be defective in the stable recruitment of chaperones. Importantly, several studies point toward a critical role for these chaperones in the quality control of Ag presentation (31, 32), a multifactorial process where tapasin in cooperation with ER60 and Crt improves loading of MHC I with high-affinity ligands. A recent working model postulates that tapasin and the chaperones optimize peptides for MHC I by deforming the peptide-binding pocket in a way that some stabilizing contacts to the peptide cannot be formed (1). MHC I molecules in this conformation load only high-affinity peptides that fulfill the requirements for stable binding. In line with this view, our results suggest that PLCs formed by 1-2ΔN lost their quality control function in MHC I loading by a failure in the normal chaperone-assisted peptide editing via tapasin.

We provide evidence that the N domains of TAP1 and TAP2 have distinct requirements for stable recruitment of ER60, Crt and Cnx (Fig. 7A) and that the N domain of TAP2 plays a key role in the proper assembly of PLCs. Cnx was reported to be a component of a TAP/tapasin-containing precursor complex that precedes the entry of MHC I and Crt (25). Thus, poor recruitment of chaperones by 1-2ΔN may reflect problems that arise early in the biosynthetic assembly of the PLC. Recent studies suggest that cooperative interactions between the chaperones are required for their stable association with the precursor complex and the PLC (25, 31–33). However, the exact molecular basis of this phenomenon is unknown and the assembly pathway of the PLC is only partly understood. Our experiments in Figs. 4 and 5 suggest that single expressed TAP1 subunits bind tapasin solely via the coreTMD whereas free TAP2 and the assembled TAP molecules use the N domains for tapasin binding. Thus, it is reasonable to speculate that pairing of the TAP subunits induces a rearrangement of tapasin from the coreTMD to the N domain of TAP1. Our own studies provide evidence that relocation of tapasin binding within TAP1 is controlled by a process where pre-existing TAP1 chains assemble with newly synthesized TAP2 (K. Keusekotten, R. M. Leonhardt, and M. Knittler, manuscript in preparation). In view of this scenario, the N domain of TAP2 might have a critical role in stabilizing the interaction with chaperones during the structural rearrangement of the tapasin-docking site within TAP1.

The expression level of TAP1 seems to control the amount of stable heterodimeric TAP (34). TAP1 expression in turn depends on the ability of tapasin to interact with TAP. This becomes evident by a dramatic decrease (up to 100-fold) in the steady state levels of TAP1 in cells that are tapasin deficient or express tapasin mutants that fail to associate with TAP (4, 7). However, the steady-state level of 1ΔN is not affected when compared with TAP1, irrespective whether it is expressed as single chain or in conjunction with TAP2 (Figs. 1B and 5A). This suggests that stabilization of TAP1 occurs via tapasin-binding to the coreTMD and therefore most likely takes place before and/or during the biosynthetic assembly of the transporter.

Repeated transfection experiments failed to generate T2 cell lines stably coexpressing 1ΔN and 2ΔN. That Koch et al. (8) observed nothing comparable in baculovirus-infected insect cells might be due to the fact that they used a transient overexpression system. Nevertheless, head-to-tail fusion of 1ΔN and 2ΔN linked by the connector region of MDR1b allows stable expression of a transporter lacking both N domains. The transport activity of the tandem TAPs (Fig. 3D) demonstrates that the fused TAP subunits can form the same functional interfaces as they do in heterodimeric TAPwt. This suggests that the structural organization and pairing of the TMDs and nucleotide-binding domains (NBVs) is highly conserved between “tandem-” and “two chain”-ATP-binding cassette transporters. The connector region of MDR1 acts as a flexible linker that allows proper folding, pairing, and coordinated interaction of the two homologous halves as mutations in this region affect protein stability and transport activity (35). Thus, although clearly not essential for the expression of single TAP chains (Fig. 5A) or the formation of a functional translocation pore (Fig. 3D), the N domains of TAP may be part of a structural unit that analogous to the connector region is critical for the efficiency of transporter biogenesis by reducing the risk of misassembly, proteolytic degradation, or accumulation of cell-toxic aggregates.

Characterization of the MHC I presentation pathway in T2(1-2ΔN) shows that in a situation where the quality control in the PLC fails stabilization of ER-exported MHC I occurs during transport to the plasma membrane (Fig. 6). Similar observations were also reported for tapasin-deficient cells (6). However, it should be noted that based on previous evidence of MHC I-specific differences in tapasin dependence, the extent and nature of post-ER stabilization may be allele-specific (36, 37). Our studies suggest that post-ER stabilization of the tapasin-dependent allele HLA-B5 requires pPC activity, but not endocytic recycling and/or endosomal processing (Fig. 7B). Low-affinity peptide ligands tend to dissociate from MHC I at acidic pH conditions corresponding to the TGN (determined to be pH 5.0) (26). Furthermore, other studies (38) indicate that at an acidic pH ~5.0 MHC I molecules are peptide-receptive and can perform efficient peptide exchange. Because pPCs, e.g., furin, are active in the TGN (9), we speculate that stabilization of suboptimally loaded MHC I occurs via exchange of bound peptides for ones that originate from the proteolytic activity of furin or furin-related proteases. However, we consider it unlikely that the peptide pool to which pPC-activity
contributes has a higher affinity for MHC I as the peptides generated by the proteasome. It is more likely that the acidic environment in the TGN confers high stringency on MHC I loading and allows only optimal peptide ligands to bind. Thus, we propose that the TGN serves as a post-ER “checkpoint” of MHC I processing that rescues unstable MHC I complexes, which otherwise have a reduced chance to survive on the cell surface. Because MHC I ligands are much shorter in length than the propeptides liberated by pPCs, further processing by other proteases would be required. Carboxypeptidases, which reside in the TGN, may function in the generation of MHC I binding peptides after processing by pPC activity (10). It is interesting to note that a plethora of viral proteins is processed by members of the pPC family (39). Because evasion strategies of some viruses target tapasin-mediated peptide optimization and cause the generation of unstable MHC I molecules (40), the furin-dependent rescue of MHC I complexes could be part of a cellular counterstrategy that undermines viral efforts to block the Ag presentation machinery.

Our current interest concentrates on the molecular mechanisms and the in vivo-relevance of the pPC-dependent MHC I presentation. So far, 7 different members of the pPC family have been identified (9). Although HDA is known to have a strong inhibitory effect on furin-activity (28) our studies (Fig. 7B) do not exclude a proteolytic contribution of other furin-related pPCs in this pathway. Of the known pPCs only four, namely furin, PC7, PC5, and PACE4 show a broad tissue distribution (9). Our own experiments show that only PC7 and furin, which can both be inhibited by polyarginines (41), are expressed at significant levels in T2 cells (data not shown). Thus, in future studies it will be of great interest to see whether furin, PC7 or both are involved in the post-ER stabilization of MHC I and what kind of peptide ligands participates in the proposed reloading process.

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Disclosures

The authors have no financial conflict of interest.

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5. Tan, P., H. Kropscholer, O. Mandelboim, N. Bulbuc, G. J. Hammerling, and F. Momburg. 2002. Recruitment of MHC class I molecules by tapasin into the TGN, may function in the generation of unstable MHC I molecules (40), the furin-dependent rescue of MHC I complexes could be part of a cellular counterstrategy that undermines viral efforts to block the Ag presentation machinery.

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