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Molecular Architecture of the TAP-Associated MHC Class I Peptide-Loading Complex

Elke Rufer, Ralf M. Leonhardt, and Michael R. Knittler

Tapasin organizes the peptide-loading complex (PLC) by recruiting peptide-receptive MHC class I (MHC-I) and accessory chaperones to the N-terminal regions of the TAP subunits TAP1 and TAP2. Despite numerous studies have shown that the formation of the PLC is essential to facilitate proper MHC-I loading, the molecular architecture of this complex is still highly controversial. We studied the stoichiometry of the PLC by blue native-PAGE in combination with Ab-shift assays and found that TAP/tapasin complexes exist at steady state as a mixture of two distinct oligomers of 350 and 450 kDa. Only the higher m.w. complex contains MHC-I and disulfide-linked tapasin/ER60 conjugates. Moreover, we show for the first time to our knowledge that the fully assembled PLC comprises two tapasin, two ER60, but only one complex of MHC-I and calreticulin. Based hereon we postulate that the TAP subunits alternate in the recruitment and loading of a single MHC-I. The Journal of Immunology, 2007, 179: 5717–5727.

The transporter associated with antigen processing (TAP) pumps proteasome-derived cytosolic peptides into the endoplasmic reticulum (ER). There, these peptides are loaded onto newly synthesized MHC class I molecules (MHC-I) that transiently associate with TAP and accessory chaperones (tapasin, calreticulin, and ER60) to form the peptide-loading complex (PLC) (1). Following peptide acquisition MHC-I dissociates from the PLC and migrates to the cell surface where it presents its ligand to CTLs.

The heterodimeric ATP-binding cassette transporter TAP comprises the two subunits TAP1 and TAP2 (2), both of which contain a transmembrane domain at their N terminus followed by a cytosolic nucleotide-binding domain. It has been shown that the N-terminal transmembrane helices (N-domains) of both TAP subunits bind independently to tapasin and provide two separate platforms for the assembly of the PLC (3, 4). Tapasin (5) plays a key role in the formation and function of the PLC as it is critically involved in the stabilization of TAP, the recruitment of MHC-I and accessory chaperones, the coordination of peptide loading onto MHC-I as well as the quality control of MHC-I loading (1). However, although much has been learned about the function of the PLC, the molecular architecture of this oligomeric complex is still controversial and not fully understood. As a matter of fact, the proposed number of tapasin and MHC-I molecules in the PLC ranges from 1 (6) to 4 (5), and it is not clear whether all tapasin docking sites in TAP are used for MHC-I binding at the same time. Additionally, our former studies suggest that the number of tapasin molecules in the PLC does not equal the number of MHC-I (4), a finding that sharply contradicts with previous models of the PLC stoichiometry (5, 6).

Given the crucial importance of the PLC arrangement for understanding the process of MHC-I loading (1), viral evasion strategies (7), and the mechanism of quality control in the loading complex (8), we have analyzed the molecular architecture of the multisubunit complex by using blue native (BN)-PAGE in combination with Ab-shift experiments. We show for the first time that TAP/tapasin complexes exist at steady state as a stable mixture of two distinct oligomers with molecular masses of 350 and 450 kDa. Only the higher m.w. complex contains MHC-I whereas the other may represent a pre- or postloading intermediate. Notably, the number of tapasin molecules equals that of ER60 molecules in both complexes, suggesting that the two polypeptides may form one functional unit. However, the previously described disulfide-linkage between tapasin and ER60 (9) was found in the 450-kDa but not in the 350-kDa TAP complex. Furthermore, in line with our former studies (4) we demonstrate that in the functional PLC, heterodimeric TAP interacts simultaneously with two tapasin molecules, but only one single MHC-I. Moreover, in support of the hypothesis that calreticulin becomes inserted into the PLC together with MHC-I, the 450-kDa TAP complex contains only one molecule of the lectin chaperone. Consequently, each TAP subunit contains a single docking site for a tapasin/ER60 dimer and at any given time only one of two TAP chains is actively involved in the tapasin-dependent recruitment of a single MHC-I/calreticulin complex. In conclusion, this study provides new insights into the structural organization of the PLC and contributes to the understanding of the early processes of MHC-I-mediated Ag processing.
Materials and Methods

Cell lines and cell culture

TAP-deficient T2 cells (10) are derivatives of the human TAP-proficient lymphoblastoid cell line T1 (10, 11). Both express HLA-A2 and HLA-B5. Stable transfectants of T2 expressing wild-type rat TAP*, TAP1–ΔN, TAP2–ΔN, and TDIΔN/ΔN (4), nontransfected T2 cells and T1 cells were cultured in IMDM/10% FCS (Invitrogen Life Technologies) supplemented with 1 mg/ml G418 (PAA Laboratories). Culture medium contained 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen Life Technologies).

Ab production

Antisera against the human and rat TAP also do not differ in their properties to interact with PLC components and do form complexes of equal size in human cells. Therefore, we performed sequential immunodepletion (Fig. 1A, top), quantitative immunodepletion (Fig. 1A, bottom) and BN-PAGE analysis (Fig. 1B) using digitonin lysates of T1 or T2 rat TAP wild-type (T2(rTAPwt)) cells (expressing rat TAP) (data not shown), suggesting that they are functionally equivalent when expressed in human cells. Thus, in our initial analysis we wanted to prove that human and rat TAP also do not differ in their properties to interact with PLC components and do form complexes of equal size in human cells. Therefore, we performed sequential immunodepletion (Fig. 1A, top), quantitative immunodepletion (Fig. 1A, bottom) and BN-PAGE analysis (Fig. 1B) using digitonin lysates of T1 or T2(rTAPwt) cells. Neither immunodepletion nor immunodepletion experiments (Fig. 1A) reveal any differences between human and rat TAP in their physical complex formation with tapasin and MHC-I. Both transporters show the same high efficiency of tapasin recruitment, and ~30–40% of ER-localized MHC-I is TAP-bound at steady state (Fig. 1A, bottom). Most importantly, BN-PAGE analysis in Fig. 1B showed that both human and rat TAP form the same two species of high m.w. complexes with molecular masses of 350 and 450 kDa, respectively. We note that these sizes are not consistent with the stoichiometry of the human PLC proposed by Ortmann et al. (5), which would demand an ~1 MDa complex (25). Quantification of the Western blot signals obtained for human and rat TAP revealed that in both cases the ratio of the 450- to 350-kDa complex is ~3. In conclusion, human and rat TAP participate in the formation of two defined high m.w. complexes of molecular mass 350 and 450 kDa.

Both populations of TAP complexes contain tapasin, but differ in the recruitment of MHC-I

To investigate whether the 350- and 450-kDa complexes, which are visible only under native cell lysis conditions (see Fig. 2, A and B), contain both TAP subunits and interact with tapasin and MHC-I, we performed BN-PAGE experiments with digitonin-lysed T1 cells and T2(rTAPwt) transfectants, in which we directly compared the electrophoretic migration behavior of protein complexes formed by TAP1, TAP2, tapasin, and MHC-I. As can be seen from Fig. 3, lanes 1 and 2, TAP1- and TAP2-containing protein complexes exhibit comparable electrophoretic migration patterns in the BN-gel showing that both transporter subunits are present in the 350-kDa as well as in the 450-kDa complex. The same could be observed for tapasin (Fig. 3, lane 3). Moreover, it seems that in both cell lines, <20% of the tapasin molecules are excluded from the two complexes and run as a smear between 80 and 180 kDa. A completely different picture was seen for MHC-I (Fig. 3, lane 4). The vast majority of MHC-I molecules is apparently “TAP complex-free” and migrates as a relatively broad smear between 70 and 160 kDa, most likely reflecting different

BN-PAGE has been used to determine the molecular composition of various membrane-localized multisubunit complexes including the B and T cell Ag receptors (20, 21) as well as the multisubunit translocon complex in the mitochondrial (22) and chloroplast membranes (23). In fact, the resolution of BN-PAGE is much higher than that of gel filtration or sucrose-gradient ultracentrifugation techniques. Furthermore, long incubation steps, which may cause partial disintegration of oligomers are avoided, rendering BN-PAGE a superior technique to analyze multiprotein complexes. Thus, in our present work we used BN-PAGE to investigate the subunit composition and stoichiometry of the TAP-associated PLC.

Protein A enrichment of antisera against ER60 and calreticulin

Antisera against ER60 (anti-ER60) and calreticulin (SPA-600; StressGen Biotechnologies) were rabbit antisera binding ER60 and calreticulin, respectively. Rabbit antisera SPA-865 and SPA-890 (StressGen Biotechnologies) are specific for calnexin and protein disulfide isomerase (PDI). HRP-conjugated Abs were purchased from Dianova.

Immunoprecipitation

Abs used for immunoprecipitation were coupled to cyanogen bromide-activated Sepharose (Sigma-Aldrich), protein A-, or protein G-Sepharose (Amersham Biosciences). In the case of anti-TAP, anti-ER60 and anti-calreticulin precipitating Abs were cross-linked to protein A or protein G by dimethylpimelimidate. For immunoisolation experiments, cells were washed in PBS before lysis in 1% digitonin (Sigma-Aldrich). For Ab-shift/BN-PAGE analysis, lysate samples were separated by SDS-PAGE and analyzed by Western blot as previously described (4).

BN-PAGE, Ab-shift analysis, SDS-PAGE, and Western blot analysis

BN-PAGE was conducted as described by Schägger et al. (19) with the following modifications. A total of 107 cells were lysed in 100 μl of BN buffer (25 mM bisTris-HCl (Sigma-Aldrich) (pH 7), 20% glycerol, containing 1% digitonin (Sigma-Aldrich). For Ab-shift/BN-PAGE experiments, lysate samples were preincubated with mAb 3B10.7 (anti-HLA), PasTa-1 (anti-tapasin), or protein A-enriched anti-ER60 or anti-calreticulin. The Ab amounts indicated were added to the lysate samples (20 μl) and incubated for 45 min on ice. Lysates incubated with or without Ab were separated by BN-PAGE (gel gradient 5.0–13.5% polyacrylamide) at 4°C. Before sample loading, 20 μl of the lysate were mixed with 2 μl of BN-sample buffer (100 mM bisTris-HCl (Sigma-Aldrich), 0.5 M 6-amino-caproic acid (Sigma-Aldrich) (pH 7.0), 30% sucrose, 50 mg ServaG (Serva-Pure)) and incubated for 10 min on ice. As protein markers, monomeric BSA (66 kDa), aldolase (158 kDa), and the 24-mer (440 kDa) were used for migration calibration (Fig. 1A, bottom). The BN-gels were run for 5–6 h. The gel run was performed with cathode buffer (50 mM Tricine, 15 mM bisTris (pH 7.0), 0.1% ServaG) and anode buffer (25 mM bisTris (pH 7.0)). The blue cathode buffer was exchanged with a colorless cathode buffer during the run after 6–8 h. For immunoblotting, the BN-gel was incubated for 10 min in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol) containing 0.1% SDS. SDS-PAGE and Western blotting were performed using standard protocols. Protein markers for BN- and SDS-PAGE were purchased from Sigma-Aldrich and Fermentas, respectively. Immunoblots were developed with specific Abs against human TAP1, human TAP2, rat TAP1, rat TAP2, ER60, PDI, calreticulin, calnexin, tapasin, and MHC-I H chain.

RESULTS

TAP participates in the formation of two species of high m.w. complexes

Stable transfectants of T2 expressing wild-type rat TAP2 (12) or rat TAP1 (13) are derivatives of the human TAP-proficient T2(rTAPwt) cells (expressing rat TAP) (data not shown), suggesting that they are functionally equivalent when expressed in human cells. Thus, in our initial analysis we wanted to prove that human and rat TAP also do not differ in their properties to interact with PLC components and do form complexes of equal size in human cells. Therefore, we performed sequential immunodepletion (Fig. 1A, top), quantitative immunodepletion (Fig. 1A, bottom) and BN-PAGE analysis (Fig. 1B) using digitonin lysates of T1 or T2(rTAPwt) cells. Neither immunodepletion nor immunodepletion experiments (Fig. 1A) reveal any differences between human and rat TAP in their physical complex formation with tapasin and MHC-I. Both transporters show the same high efficiency of tapasin recruitment, and ~30–40% of ER-localized MHC-I is TAP-bound at steady state (Fig. 1A, bottom). Most importantly, BN-PAGE analysis in Fig. 1B showed that both human and rat TAP form the same two species of high m.w. complexes with molecular masses of 350 and 450 kDa, respectively. We note that these sizes are not consistent with the stoichiometry of the human PLC proposed by Ortmann et al. (5), which would demand an ~1 MDa complex (25). Quantification of the Western blot signals obtained for human and rat TAP revealed that in both cases the ratio of the 450- to 350-kDa complex is ~3. In conclusion, human and rat TAP participate in the formation of two defined high m.w. complexes of molecular mass 350 and 450 kDa.

Both populations of TAP complexes contain tapasin, but differ in the recruitment of MHC-I
intracellular maturation forms of MHC-I. The MHC-I migration profile obtained by densitometric scanning (Fig. 3, right panels) revealed that at steady state, ~10–15% of all MHC-I molecules are associated with the 450-kDa complex. Strikingly, however, no MHC-I at all could be detected in the smaller 350-kDa complex. Taken together, we conclude that two distinct species of TAP/tapasin complexes exist in the ER membrane, which are a 350-kDa complex lacking and a 450-kDa complex comprising MHC-I.

In the fully assembled PLC, heterodimeric TAP interacts with two tapasins, but only with one MHC-I molecule

As mentioned, based on the studies of Ortmann et al. (5), it was calculated that the PLC would have a molecular mass of ~1 MDa (25). However, using the same stabilizing detergent conditions as Ortmann et al. (5), our BN-PAGE experiments (Figs. 1 and 2) identified solely two TAP complexes that are substantially smaller in size. This suggests that the PLC in fact contains less than the postulated four MHC-I and four tapasin molecules (5).

To ascertain the molecular architecture of the two identified TAP complexes and the number of PLC-bound MHC-I and tapasin molecules, we performed Ab-shift/BN-PAGE experiments, which have been used to determine the composition of other membrane-bound multisubunit protein complexes (21). We used for our studies the purified mAbs 3B10.7 (rat anti-MHC-I) (16) and PasTa-1 (mouse anti-tapasin) (9). Both mAbs are ideal for Ab-shift/BN-PAGE experiments as both form stably assembled Ig H2L2 tetramers (Fig. 4A, left), migrate in the native BN-gel as single well-resolved protein complexes of ~170 kDa (Fig. 4B, right) and recognize their Ags in TAP-associated PLCs (4, 9).

By using sequential immunoprecipitation (Fig. 4B), we first tested whether the Ab binding of complex-associated MHC-I and tapasin occurs in a quantitative manner. Therefore, anti-TAP-precipitated complexes (Fig. 4B, lanes 1 and 5) were incubated with 3B10.7 or PasTa-1. After extensive washing of the Ab-treated precipitates, MHC-I and tapasin were dissociated from the isolated TAP complexes by incubation with destabilizing detergents and then re-isolated by consecutive immunoisolations using protein G (Fig. 4B, lanes 2, 3, 6, and 7, first and second round...
of reprecipitation), anti-MHC-I and anti-tapasin (Fig. 4B, lanes 4 and 8, third round of reprecipitation). As can be seen from Fig. 4B, MHC-I and tapasin were substantially recovered in the first and second round of re-isolation with protein G, whereas no isolated molecules were detected in the third round of reprecipitation with anti-MHC-I and anti-tapasin. This finding indicates that all MHC-I and tapasin molecules present in the TAP complex were recognized and bound by 3B10.7 and PasTa-1. Moreover, the reprecipitation steps with protein G suggest that the amount of bound anti-MHC-I and anti-tapasin Abs equals the amount of re-isolated MHC-I and tapasin, suggesting a stoichiometric 1:1-interaction between the Abs and complex-bound Ags.

For the Ab-shift analysis, digitonin lysates of T2(rTAPwt) cells were first preincubated with increasing amounts of anti-MHC-I or anti-tapasin, subjected to BN-PAGE and analyzed by anti-TAP immunoblots (Fig. 4C). Ab binding to the TAP complex is reflected by the appearance of shifted bands (Fig. 4C, lanes 7, 8, 14, 15, and 16). In the case of the anti-MHC-I shift analysis (Fig. 4C, lanes 1–8) we observed, in the presence of 10^{-2} and 10^{-1} μg of mAb, a single 170-kDa shift for the 450-kDa complex, whereas in agreement with the findings in Fig. 3, no anti-MHC-I shift was produced for the 350-kDa complex (Fig. 4C, lane 7). The same electrophoretic band pattern, but with a quantitative shift of the complex, was observed when the amount of anti-MHC-I was increased to 1 μg (Fig. 4C, lane 8). In additional control experiments, we checked the binding of the Ab to the 450-kDa complex by restaining the last three tracks of the anti-TAP blot (Fig. 4C, lanes 7 and 8, 10^{-2}–1 μg of anti-MHC-I) with anti-rat IgG-HRP (Fig. 4D, left). This confirmed that the 620-kDa shift product of the 450-kDa complex was produced by the binding of 3B10.7 and that the observed single shift was complete in the presence of Ab excess (1 μg of 3B10.7). Taken together, data show that only a single MHC-I molecule is present in the 450-kDa complex, whereas none is present in the 350-kDa complex.

In the Ab-shift/BN-PAGE experiments performed with antitapasin (Fig. 4C, lanes 9–16), we found that after preincubation with 10^{-2} μg of Ab both TAP complexes were shifted by 170 kDa (Fig. 4C, lane 14). When the amount of Ab was increased to 10^{-1} μg, a 340-kDa shift was observed for the 350-kDa as well as for the 450-kDa complex (Fig. 4C, lane 15). No further Ab-shift of the two complexes was detected in the presence of 1 μg anti-tapasin Ab (Fig. 4C, lane 16). The corresponding control Western blots stained with anti-mouse IgG-HRP (Fig. 4D, right) verified that anti-tapasin binding produced the first and second set of shifts and that the latter shift of the two complexes was complete in the presence of Ab excess (1 μg of PasTA-1).

Thus, in agreement with the results shown in Fig. 3, both TAP-containing complexes comprise two tapasin molecules, whereas solely the 450-kDa complex contains a single MHC-I.

TAP variants lacking the tapasin docking sites in TAP2 or TAP1 show MHC-I interaction comparable to that of the wild-type transporter

In a former study we had analyzed the functional properties of truncated TAP variants lacking the tapasin docking sites in either TAP1 or TAP2 (4). Most interestingly, although both TAP mutants displayed the expected reduction of tapasin binding by 50%, we found that comparable amounts of MHC-I bound and passed through the PLCs formed by wild-type TAP or its two deletion variants (4). In view of this finding, and the results of the Ab-shift/BN-PAGE experiments performed with anti-MHC-I (Fig. 4C, lanes 9–16), we found that after preincubation with 10^{-2} μg of Ab both TAP complexes were shifted by 170 kDa (Fig. 4C, lane 14). When the amount of Ab was increased to 10^{-1} μg, a 340-kDa shift was observed for the 350-kDa as well as for the 450-kDa complex (Fig. 4C, lane 15). No further Ab-shift of the two complexes was detected in the presence of 1 μg anti-tapasin Ab (Fig. 4C, lane 16). The corresponding control Western blots stained with anti-mouse IgG-HRP (Fig. 4D, right) verified that anti-tapasin binding produced the first and second set of shifts and that the latter shift of the two complexes was complete in the presence of Ab excess (1 μg of PasTA-1).

Thus, in agreement with the results shown in Fig. 3, both TAP-containing complexes comprise two tapasin molecules, whereas solely the 450-kDa complex contains a single MHC-I.

To verify this speculation, we analyzed the molecular architecture of TAP complexes comprising TAP deprived of the tapasin docking site in TAP1 (TAP2-1ΔN), TAP2 (TAP1–2ΔN), or in both subunits (TD1ΔN/2ΔN) (4). The BN-PAGE experiment shown in Fig. 5A, left, lane 2, revealed that variant TAP2-1ΔN forms two complexes with molecular masses of 230 and 330 kDa. Quantitation of the obtained signals by densiometrical scanning (Fig. 5A, right) revealed a ratio between the two complex populations of around 1.5 to 2. Interestingly, TAP1–2ΔN also participated in a 230- and 330-kDa complex, suggesting that the molecular composition of PLCs formed by the two TAP deletion variants is identical. However, in support of our previous observation that TAP variants lacking the tapasin docking site in TAP2 are disturbed in the stable incorporation of accessory chaperones into the PLC (4), an additional complex species of 180 kDa could be detected for TAP1–2ΔN (Fig. 5A, left, lane 3) probably reflecting a product of partial complex disintegration. We observed that the relative amount of the 180-kDa species increases significantly when lysate samples of T2(TAP1–2ΔN) are analyzed by BN-PAGE after extended preincubation (Fig. 5A, right). In contrast to TAP2-1ΔN and TAP1–2ΔN, the transporter variant TD1ΔN/2ΔN, which lacks both tapasin docking sites, migrated in the BN-gel as
FIGURE 4. Number of tapasin and MHC-I molecules in the assembled PLC. A, Characterization of anti-MHC-I (3B10.7) and anti-tapasin (PasTa-1) Abs used for Ab-shift/BN-PAGE experiments. Purified Abs were separated by SDS-PAGE (left), reducing conditions (R), nonreducing conditions (NR), or BN-PAGE (right) and then analyzed by Coomassie staining. Western blots were probed for rat or mouse IgG. B, Anti-MHC-I (3B10.7) and anti-tapasin (PasTa-1) bind efficiently to their Ags in the TAP-associated PLC. TAP complexes were immunoisolated from digitonin lysates of T2(rTAPwt) cells with anti-TAP2 Ab (Mac394) (lanes 1 and 5) and subsequently incubated with anti-MHC-I or anti-tapasin. Unbound Abs were removed from the samples by extensive washing with PBS/0.1% digitonin. To dissociate MHC-I and tapasin (TPN) from the isolated TAP complexes, the precipitates were incubated with PBS/1% Triton X-100/1% Nonidet P-40. Finally, supernatants were separated by centrifugation and subjected to sequential immunoprecipitation (IP) with protein G-Sepharose (1.IP, lanes 2 and 6; 2.IP, lanes 3 and 7) or to anti-MHC-I-Sepharose or anti-tapasin-Sepharose (3.IP, lanes 4 and 8). The different immunoisolates were separated by nonreducing gel electrophoresis and analyzed in Western blots probed for both TAP chains, MHC-I, and tapasin (TPN). In case of protein G, isolated Abs were visualized by costaining the anti-MHC-I and anti-tapasin blots with anti-rat IgG-HRP and anti-mouse IgG-HRP, respectively. C, Ab-shift/BN-PAGE analysis of the PLC with anti-MHC-I and anti-tapasin Abs. Lysate samples of T2(rTAPwt) were incubated with increasing amounts of anti-MHC-I (lanes 1–8) and anti-tapasin Abs (lanes 9–16). After incubation for 45 min in the cold, lysates were separated by BN-PAGE and analyzed in Western blots stained with a mixture of Abs specific for rat TAP1 and TAP2. Complexes and Ab-shift products are indicated. D, Specific Ab binding to the PLC. The last three tracks (preincubation with $10^{-2}$–1 μg of Ab (lanes 6–8 and 14–16) of the immunoblots in C were stripped and reprobed with anti-rat IgG-HRP (left) and anti-mouse IgG-HRP (right).
a single species with a molecular mass of 110 kDa. Thus, presence
and number of tapasin docking-sites in the different TAP variants
correlate with the formation and molecular mass of the identified
TAP complexes.

We next performed Ab-shift/BN-PAGE experiments with
TAP2-1ΔN, TAP1–2ΔN, and TD1ΔN/2ΔN. Therefore, digitonin-
lysates of the respective T2 transfectants were incubated with in-
creasing amounts of anti-MHC-I and anti-tapasin, subjected to
BN-PAGE, and analyzed by anti-MHC-I and anti-tapasin Abs.
Assembled PLCs and dissociated TAP complexes are indicated. B
and C. The number of tapasin (TPN) and MHC-I molecules in the PLCs formed by
TAP1–2ΔN and TAP2-1ΔN. For the Ab-shift/BN-PAGE analyses, lysate samples of the T2 transfectants were incubated with increasing amounts of
anti-MHC-I and anti-tapasin Abs and analyzed as described in Fig. 4C, D. Ab-shift/BN-PAGE assay using cell extracts of T2(TD1ΔN/2ΔN), TAP
complexes and Ab-shift products are indicated.

FIGURE 5. Composition of PLCs lacking the tapasin docking site in TAP1 or TAP2. A, Complex formation by TAPwt, TAP1–2ΔN, TAP2-1ΔN, and
tDP2/1ΔN/2ΔN (left and middle). T2 transfectants were lysed in complex stabilizing BN buffer containing 1% digitonin. Lysates were separated by BN-PAGE
and then analyzed in Western blots probed for rat TAP1 and TAP2 (left). The additional complex species of TAP1–2ΔN (+) is indicated. For signal
quantitation, the different tracks were scanned in a linear mode by microdensitometry (middle). Analysis of TAP complex stability in cell extracts of
T2(TAP2-1ΔN) and T2(TAP1–2ΔN) (right). T2 transfectants (T2(TAP2-1ΔN) and T2(TAP1–2ΔN)) were lysed in BN buffer containing 1% digitonin.
Lysates were first preincubated for 0, 6, 12, and 24 h on a overhead-rotator and then electrophoretically separated by BN-PAGE. After protein
transfer onto nitrocellulose, the Western blot membranes were immunostained with a 1:1 mixture of anti-rat TAPI (D90) and anti-rat TAP2 (116/5) Abs.
Assembled PLCs and dissociated TAP complexes are indicated. B and C. The number of tapasin (TPN) and MHC-I molecules in the PLCs formed by
TAP1–2ΔN and TAP2-1ΔN. For the Ab-shift/BN-PAGE analyses, lysate samples of the T2 transfectants were incubated with increasing amounts of
anti-MHC-I and anti-tapasin Abs and analyzed as described in Fig. 4C, D. Ab-shift/BN-PAGE assay using cell extracts of T2(TD1ΔN/2ΔN), TAP
complexes and Ab-shift products are indicated.

TAP (Fig. 4C), no second shift of the two TAP complexes was
produced in the presence of 1 μg of anti-tapasin. Thus, only one
molecule of tapasin is present in each of the two truncated TAP
complexes. We note that the additional 180-kDa complex species
of TAP1–2ΔN might have been also affected by preincubation
with anti-MHC-I and anti-tapasin, although in this case no clear
corresponding shift products could be identified in the BN-gel.
Nevertheless, that the identified 170- and 340-kDa shifts of the
different TAP complexes (Figs. 4C and 5, B and C) are indeed
produced by the specific Ag binding of anti-MHC-I and anti-
tapasin could be directly seen in the experiments with variant
TD1ΔN/2ΔN, which does not form complexes with tapasin and
MHC-I at all (4). Preincubation of cell lysates from T2(TD1ΔN/2ΔN)
with 1 μg of anti-MHC-I or anti-tapasin or both did not
produce a detectable change in the electrophoretic migration be-
havior of the identified 110-kDa species (Fig. 5D).

Thus, wild-type and truncated TAPs do not interact with more
than a single MHC-I. However, they differ in the number of bound
tapasin. Based on this difference and the observed steady state ratio
of “MHC-I-containing” and “MHC-I-free” TAP/tapasin complexes, we postulate an alternating docking site mechanism in the PLC, in which the two TAP-associated tapasin molecules normally alternate with each other in the recruitment of a single newly synthesized MHC-I molecule.

The TAP-associated PLC contains a single calreticulin and two molecules of ER60

Previous studies have suggested that all TAP-associated ER60 molecules are conjugated to tapasin (9) and that the assembled functional PLC results from the introduction of calreticulin-associated MHC-I (26).

To analyze whether the number of these accessory chaperones corresponds to the number of complex-bound tapasin and MHC-I molecules, we performed Ab-shift/BN-PAGE experiments using anti-ER60 and anti-calreticulin Abs. Both Abs migrate in the native BN-gels as defined protein complexes (Fig. 6A) and recognize the native forms of the chaperones in the TAP-associated PLC (see Fig. 6B) (27, 28). Digitonin lysates of T2(rTAPwt) were incubated with increasing amounts of anti-ER60 and anti-calreticulin Abs. After incubation for 45 min on ice, the lysates were separated by BN-PAGE and then analyzed in Western blots stained with a mixture of antiserum specific for rat TAP1 and TAP2. TAP complexes and Ab-shift products are indicated. The unresolved smear at the top of the BN-gels (**) is most likely due to nonspecific protein aggregation or overload in the presence of high Ab concentrations. D, TAP complex interaction of calnexin (CNX) and PDI. Digitonin lysates of T2(rTAPwt) were subjected to immunoprecipitation using the anti-rat TAP2 antiserum (116/5). Subsequently, bound protein complexes were eluted by competition with a peptide corresponding to the C terminus of TAP2 (EQDYAHLVQQRLEA, in single letter amino acid code). The mildly eluted TAP complexes were separated by two-dimensional BN-PAGE or SDS-PAGE, in which the material was separated by first-dimension BN-PAGE (top) followed by second-dimension reducing SDS-PAGE (bottom left). After protein transfer onto nitrocellulose, the membranes were immunostained with anti-calnexin, anti-PDI, or anti-tapasin Abs. In the control experiment (right), the same immunoreagents were used for Western blot analysis of lysate proteins separated by reducing SDS-PAGE. E, Disulfide-linked tapasin/ER60 dimer formation within the TAP-associated complexes. T2 transfectants expressing TAPwt were lysed in BN buffer containing 1% digitonin in the presence or absence of 10 mM sulfhydryl-reactive agent NEM. Lysate samples were separated by BN-PAGE (+NEM) or by nonreducing SDS-PAGE (+NEM or −NEM) (right bottom panels) and analyzed in Western blots stained with antisera specific for rat TAP (BN-PAGE) or tapasin and ER60 (SDS-PAGE). For two-dimensional BN-gel and SDS-PAGE analysis of the NEM-treated lysate sample, a second lane of the first-dimension BN-gel (top) was cut out and equilibrated for 30 min in nonreducing SDS loading buffer. Finally, the gel strip was placed into an SDS-gel (+NEM) (bottom left panel), and the second-dimension SDS-PAGE run was performed by a standard protocol. Finally, the proteins were transferred onto nitrocellulose membranes and tapasin was detected by immunostaining. Tapasin/ER60 dimers and monomeric forms are indicated.
of a single calreticulin in this oligomer. However, no detectable band shift in the BN-gel was observed for the 350-kDa complex (Fig. 6C, left, lane 6), demonstrating the absence of the lectin chaperone from the lower m.w. complex. In contrast to anti-calreticulin, anti-ER60 generated mobility shifts for the 450-kDa chaperone from the lower m.w. complex. In contrast to anti-

**Discussion**

The formation of the PLC is essential for the transfer of high affinity peptides onto ER-resident MHC-I and thus for the proper presentation of Ags to CTLs (1). None of the PLC components is dispensable for the generation of stable MHC-I. Consequently, many viruses attempt to disrupt or block the PLC function (7), while tumor cells frequently down-modulate the expression of its components to evade recognition (32). In the light of this mechanism, the gain of knowledge on the molecular architecture and composition of this complex is an inevitable step to fully understanding the early events in peptide selection and loading that eventually lead to the formation of stable surface MHC-I and hence an appropriate immune response. However, the stoichiometry of the PLC is still highly controversial and different structural models have been suggested. Based on velocity sedimentation experiments of cell extracts, Li et al. (6) had hypothesized that the PLC might contain only a single docking site for tapasin and MHC-I. However, this sharply contradicts with the finding that both TAP1 and TAP2 independently provide interaction sites for tapasin and that both docking sites in the transporter are indeed occupied by tapasin simultaneously (3, 4, 33). By using immunoprecipitation in combination with SDS-PAGE and amino acid analysis, Ortmann et al. (5) had postulated that the TAP-associated PLC would bind four tapasin and four MHC-I molecules at the same time. The same research group showed that tapasin does not form dimers, trimers or tetramers (34). Moreover, a current study demonstrates that in assembled TAP tapasin binding is exclusively mediated by the first N-terminal transmembrane helix of TAP1 and TAP2, respectively (3). In this light, it may be difficult to reconcile how four separate tapasin/MHC subcomplexes, all additionally associated with accessory chaperons, can be structurally grouped around the limited interaction area provided by the docking sites within two transmembrane helices, although we cannot formally exclude such a structural arrangement.

To resolve these issues we performed BN-PAGE and Ab-shift/BN-PAGE experiments, which have been successfully used for the stoichiometric characterization of diverse membrane-bound multisubunit complexes (20–23). We chose this method as it is much less prone to partial complex disintegration than immunoprecipitation-based methods and provides an excellent resolution in the separation of high m.w. complexes. We identified under stabilizing cell lysis conditions two species of TAP/tapasin complexes of 350- and 450-kDa (Fig. 1). Most interestingly, no other high m.w. complexes were detected for human or rat TAP, suggesting that the two transporter molecules do not differ in the structural formation of the PLC. Neither “free” TAP subunits nor “complex-free” transporter molecules were found (Figs. 2 and 3). Clearly, MHC-I participates in the 450-kDa TAP/tapasin complex, but is completely

The two identified TAP complexes differ in the recruitment of calnexin and the formation of disulfide-linked tapasin and ER60 dimers

The chaperone calnexin was suggested to be a component of a putative precursor TAP complex that precedes the entry of MHC-I and calreticulin into the PLC (26). Furthermore, recent reports also provide evidence that the PDI is involved in the formation of the PLC (29, 30), whereas other data do not support physical interaction of PDI with the TAP-associated loading complex (31). Unfortunately, by using Ab-shift/BN-PAGE experiments, we could not detect any physical interaction of calnexin and PDI with one of the two identified TAP complexes (data not shown). This interaction might be undetected due to the fact that the Ab epitope in calnexin and PDI is masked when bound to the PLC. Therefore, we used another experimental approach in which immunopurified TAP complexes were analyzed by two-dimensional BN-PAGE and SDS-PAGE in combination with immunoblotting. The isolated material was separated by first-dimensional BN-PAGE (Fig. 6D, upper panel) followed by second-dimension-reducing SDS-PAGE (Fig. 6D, second, third, and fourth panels on left). The results in Fig. 6D show that calnexin is an accessory chaperone of the 350-kDa complex but not of the larger 450-kDa complex. Moreover, in support of the findings of Peaper et al. (31), we did not observe physical interaction of PDI with either of the two complex species. Thus, we conclude that the 350-and 450-kDa TAP complex differ in the binding of calnexin and apparently do not contain PDI.

In a previous study, Dick and coworkers (9) showed that in the presence of the sulfhydryl-reactive agent N-ethylmaleimide (NEM), which protects native disulfide bonds during cell lysis, a significant amount of tapasin is disulfide-linked in a 1:1 ratio to ER60. To analyze dimerization of tapasin and ER60 in the two identified TAP complexes (Fig. 2), we performed two-dimensional BN-PAGE and SDS-PAGE experiments with NEM-treated cell extracts of T2(TAPwt). The cells were solubilized in BN lysis buffer in the presence and absence of 10 mM NEM. Proteins in the different samples were separated by nonreducing SDS-PAGE (Fig. 6E, bottom right) and two-dimensional BN-PAGE or SDS-PAGE (Fig. 6E, top and left bottom) and analyzed by immunoblotting. In agreement with previous observations (9, 31), immunostaining of the nonreducing SDS-PAGE membranes with anti-tapasin and anti-ER60 (Fig. 6E, bottom right) identified in NEM-treated lysates tapasin and ER60 as components of the characteristic 110-kDa dimer, whereas no such conjugates were seen in the absence of NEM. Separation of the NEM-treated lysate sample by BN-PAGE (Fig. 6E, top) showed that the reagent has no effect on the electrophoretic migration behavior of the two identified TAP complexes, suggesting that the molecular composition of the two PLC species is not affected by disulfide bond protection. For further separation of the NEM-stabilized lysate in a second-dimension nonreducing SDS-PAGE (Fig. 6E, bottom left) a second lane from the first-dimension BN-PAGE (corresponding to Fig. 6E, top) was cut out, and placed into a nonreducing SDS-gel. Immunostaining of the two-dimensional BN-PAGE and SDS-PAGE membrane with anti-tapasin antiserum revealed that at steady state a substantial fraction (40%) of the 450-kDa TAP complex contains disulfide-linked tapasin/ER60 dimers. Formation of these conjugates was also observed for the fraction of “free” tapasin molecules, which is excluded from the PLCs. However, in contrast, no tapasin/ER60 dimers were detected for the 350-kDa complex species.

Thus, although both types of TAP complexes recruit the same number of tapasin and ER60 molecules (Figs. 4 and 6C), the two PLC species differ in the physical interaction of the two components.
absent from the 350-kDa oligomer (Figs. 3 and 4). Our own experience (27) and that of other researchers (35) is that TAP/tapasin/MHC-I complexes are remarkably stable in the presence of mild detergents. We therefore assume that the two identified TAP/tapasin complexes reflect a snapshot of the in vivo situation and that at steady state the 450- and 350-kDa complex species are present in the ER membrane in a ratio of ~3:1. Thus, in support of the crucial function of the PLC for the Ag processing machinery (28), it seems that in the cell the majority of the TAP/tapasin complexes is physically involved in the recruitment and loading of MHC-I molecules.

In accordance with the rather moderate molecular masses of the two identified TAP/tapasin complexes, we found that the number of TAP-bound tapasin and MHC-I is much lower than the number of tapasin that has been postulated by Ortmann et al. (35), who used an immunoprecipitation-based approach to analyze the stoichiometry of the PLC. Their overestimation of the number of tapasin and MHC-I molecules in the PLC vs TAP might be explained by partial, nonquantitative elution of TAP from the affinity columns used or by copurification of significant amounts of contaminants (e.g., actin together with MHC-I). This might particularly be a problem when immunoprecipitations are conducted with mild detergents. We therefore assume that the two identified TAP/tapasin complexes that interact with two tapasin molecules, and that the 450-kDa complex species recruits only one MHC-I. Thus, although TAP theoretically has the capability to bind and load MHC-I via both transporter subunits, it seems that in the assembled PLC at any given time only one of two bound tapasin molecules interacts with a single MHC-I. Proteasomal inhibition did not increase the levels of TAP-associated MHC-I (data not shown), suggesting that binding of a single MHC-I to the PLC does not simply reflect a high MHC-I export rate in the presence of oversaturating peptide concentrations in the ER. In further support of this suggestion, we found that the transport active TAP variants TAP1–2ΔN and TAP2–1ΔN, which are deprived of the N-terminal tapasin docking sites in TAP2 or TAP1, form complexes that interact with only one tapasin molecule, but retain the ability of the wild-type PLC to bind a single MHC-I (Fig. 5, B and C). Moreover, we note that the relative amount of MHC-I-free complexes at steady state is detectably increased when TAP contains only one of two N-domains for tapasin binding (Figs. 2–5). This suggests a functional relationship between TAP/tapasin and TAP/tapasin/MHC-I complexes and provides evidence for an alternating docking site/mechanism in the wild-type PLC, in which the two TAP subunits normally alternate with each other in the recruitment of MHC-I.

Tapasin, is believed to play a key role in the formation and function of the PLC (36) because it organizes the physical interaction between TAP and MHC-I and tethers accessory chaperones into the complex. Most importantly, loading of MHC-I with peptide Ags conferring high stability requires the tapasin-mediated introduction of TAP into the PLC (28). Although it has been shown that the first N-terminal transmembrane helices of TAP1 and TAP2 functions as tapasin docking site (3), it is still unknown what kind of sequence motif or residues mediate the physical interaction with tapasin. Momburg and colleagues (28, 37) showed that mutations interrupting a putative transmembrane leucine-zipper motif in the transmembrane helix of tapasin affect the formation of TAP/tapasin complexes and the stabilization of single expressed TAP2 chains. Therefore, it was speculated by this group that such a motif might play a role in the complex formation between tapasin and TAP (28, 37). In view of this speculation and our own findings on the architecture of the PLC, it is interesting to note that the first N-terminal transmembrane helix of both transporter subunits contains a leucine-rich sequence stretch that could serve as complementary interaction site for the supposed leucine zipper of singly bound tapasin. Clearly, detailed mutational studies on the sequence of the tapasin docking site of TAP1 and TAP2 are required to resolve the molecular and structural basis of TAP/tapasin-assembly in the PLC.

The findings of our work contradict with a former model of the PLC that argued simultaneous binding of multiple MHC-I molecules would favor the association and loading of different MHC-I alleles (5). What might be the benefit of a PLC that recruits only a single MHC-I? Several viruses considerably interfere with the proper function of the peptide transporter or the PLC (7), which must be regarded as a kind of bottleneck in the MHC-I-mediated Ag presentation pathway. Thus, under conditions of a viral infection one could speculate that it would be useful for the cell to increase the number of transporter molecules to titrate away viral effectors or at least to temporarily defer a complete viral block of peptide delivery into the ER. This method would be consistent with the induction of TAP by the inflammatory cytokine IFN-γ (38). Following this argument, it might be a more promising strategy for the Ag processing machinery to provide a huge number of TAP-associated PLCs each associated with one MHC-I than to assemble complexes that comprise a high number of MHC-I molecules bound by a single TAP, which in the case of a functional block would render all bound MHC-I molecules trapped and hence useless for the immune response.

During the assembly of the PLC, tapasin and MHC-I interact with the lectin chaperone calreticulin (17) and the multifunctional oxidoeductase ER60 (9). Tapasin is essential for the physical recruitment of ER60 into the TAP-associated complex where both form an intermolecular disulfide bridge (9). In turn, calreticulin seems to be inserted into the complex in conjunction with peptide-receptive MHC-I (26). In line with these observations and the number of tapasin and MHC-I molecules that we detected in the different complex species (Fig. 4), we could show that both TAP complexes, the 350- and the 450-kDa complex, interact with two molecules of ER60, whereas only the higher m.w. MHC-I-containing complex comprises a single calreticulin (Fig. 6). We note that the molecular mass of a PLC containing one TAP, two tapasin, two ER60, one MHC-I, and one calreticulin can be estimated between 450 and 470 kDa, which correlates well with the observed electrophoretic migration behavior of the larger of the two identified TAP complexes in BN-PAGE (Figs. 1–4).

Several recent studies point toward a critical role for ER60 and calreticulin in the quality control of Ag presentation (9, 39). It was speculated that the optimization of peptide cargo for MHC-I is a multifactorial process in which tapasin in cooperation with calreticulin and ER60 alters the conformation of the peptide-binding pocket in MHC-I and thereby improves the efficient loading with optimal peptide ligands that confer high conformational stability. Calreticulin might be also involved in the cooperative stabilization of the PLC, chaperoned peptide delivery from TAP to the PLC and assisted folding of MHC-I (39). Another idea suggests that calreticulin tethers empty or suboptimally loaded MHC-I that is about to escape the ER back into the PLC (39). Such a role in MHC-I retention is in keeping with our results on the stoichiometry of the PLC (Figs. 3 and 5) and the observation that cells deficient for calreticulin display accelerated export of unstable peptide-receptive MHC-I, which largely dissociates during or upon the migration to the plasma membrane (39). In cells expressing a tapasin variant that fails to recruit ER60, MHC-I displays substantial instability, demonstrating that ER60 is an essential component of the quality control machinery that directs the loading of MHC-I with
FIGURE 7. Model of the molecular architecture and function of the PLC. We propose that the 450-kDa species of the two identified TAP complexes represents the fully assembled PLC. In this complex, TAP binds two tapasin, two ER60, and one subcomplex containing MHC-I and calreticulin (Clr) (I). Our model suggests that at any given time, only one of the two TAP subunits is actively involved in the tapasin-mediated recruitment and chaperone-assisted loading of single MHC-I molecule. After successful peptide loading the MHC-I molecule is released from the PLC (II) and a MHC-I-free complex is formed (III). We assume that this transient stage corresponds to the calnexin (CNX)-interacting 350-kDa TAP complex, in which tapasin and ER60 are not disulfide-linked to each other. The “used” MHC-I docking site may undergo a recycling process. At this time, a peptide-receptive calreticulin-bound MHC-I interacts with the tapasin/ER60 subcomplex on the opposite docking site of the PLC (IV). By continuation of this scenario the two subunits of TAP alternate with each other in the recruitment of MHC-I.

We found that the 350-kDa TAP complex interacts with calnexin (Fig. 6D) supporting the notion that the chaperone is component of a TAP complex species that precedes the physical recruitment of MHC-I and calreticulin (26). Unfortunately, we could not determine the number of calnexin molecules in the 350-kDa TAP complex. However, with respect to the calculated molecular mass of the chaperone of ~67 kDa, it is tempting to speculate that only a single calnexin molecule is present in the lower m.w. complex. In line with previous studies (26), it is reasonable to assume that calnexin could play a functional role in the transient stabilization of the 350-kDa TAP complex or the cooperative assembly of the functional PLC. In agreement with the findings of Peaper et al. (31), we could not detect PDI in the two identified TAP complexes. Santos et al (30) reported that the interaction of PDI with the PLC was not consistent in all their experiments. They suggested that PDI might be a more transient visitor to the TAP complexes with a relatively low-affinity interaction. Moreover, Park et al. (29) observed that a substantial fraction of PDI/MHC-I complexes exists outside the assembled PLC. In view of these results and our own data, the physical recruitment of PDI to the PLC remains obscure and requires further experimental characterization.

What might be the function of the 350-kDa TAP complex? Most interestingly, in contrast to the fully assembled PLC, the MHC-I-free 350-kDa complex does not contain disulfide-linked ER60/tapasin dimers (Fig. 6E), suggesting that two identified TAP complexes differ in the physical interaction and most probably the redox states of ER60 and tapasin. Thus, in view of the postulated function of ER60 as isomerase or reductase (40) regulating the α5-disulfide bonding of MHC-I H chain (41) and the idea that this activity is controlled in the PLC by tapasin (42), we speculate that the 350-kDa TAP complex could be functionally involved in the recycling of the redox-active state of ER60 after the release of the fully assembled MHC-I.

A former report from our lab describing the functional properties of ATP-binding mutants of TAP1 and TAP2 had suggested that the release of loaded MHC-I from the PLC might be directly linked to the ATP hydrolysis cycle of the transporter, particularly to nucleotide hydrolysis by TAP1 (43). The use of these mutants or viral TAP inhibitors like US6 (44), which affect the nucleotide binding in TAP1 (45), may prove very useful in future studies to further underscore this idea. Because of our recent results, we additionally note that conformational rearrangements within TAP driven by ATP hydrolysis might also direct the association of MHC-I with only one of the two docking sites provided by the PLC, whereas the opposing docking site is kept inaccessible at the same time.

Taken together, our study provides new insights into structural and functional organization of the PLC that extend our knowledge on the early events of the Ag processing machinery and might be helpful to understand viral evasion strategies directed at the biogenesis and function of TAP-associated complexes.

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