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Three Tapasin Docking Sites in TAP Cooperate To Facilitate Transporter Stabilization and Heterodimerization

Ralf M. Leonhardt,*† Parwiz Abrahimi, † Susan M. Mitchell, † and Peter Cresswell*†‡

The TAP translocates peptide Ags into the lumen of the endoplasmic reticulum for loading onto MHC class I molecules. MHC class I acquires its peptide cargo in the peptide loading complex, an oligomeric complex that the chaperone tapasin organizes by bridging TAP to MHC class I and recruiting accessory molecules such as ERp57 and calreticulin. Three tapasin binding sites on TAP have been described, two of which are located in the N-terminal domains of TAP1 and TAP2. The third binding site is present in the core transmembrane (TM) domain of TAP1 and is used only by the unassembled subunits. Tapasin is required to promote TAP stability, but through which binding site(s) it is acting is unknown. In particular, the role of tapasin binding to the core TM domain of TAP1 single chains is mysterious because this interaction is lost upon TAP2 association. In this study, we map the respective binding site in TAP1 to the polar face of the amphipathic TM helix TM9 and identify key residues that are essential to establish the interaction. We find that this interaction is dispensable for the peptide transport function but essential to achieve full stability of human TAP1. The interaction is also required for proper heterodimerization of the transporter. Based on similar results obtained using TAP mutants that lack tapasin binding to either N-terminal domain, we conclude that all three tapasin-binding sites in TAP cooperate to achieve high transporter stability and efficient heterodimerization. The Journal of Immunology, 2014, 192: 2480–2494.

Major histocompatibility complex class I–mediated Ag presentation is a major pathway to eradicate tumors and virally infected cells in the body (1, 2). To this end, peptide Ags are generated in the cytosol mostly by the proteasome (3). These are then translocated into the endoplasmic reticulum (ER) by the peptide TAP and loaded onto MHC class I molecules in the so-called peptide loading complex (PLC) (4). The PLC is organized by the chaperone tapasin (5), which simultaneously binds TAP via its transmembrane domain (TMD) (6–8) and MHC class I via its ER-lumenal domain (9). The function of the PLC is to facilitate the transfer of peptide Ags, typically 8–11 aa long, into the peptide-binding groove of MHC class I and to edit the respective peptide repertoire in a way that only high-affinity ligands are loaded (10–15). Once MHC class I has captured an appropriate ligand, it dissociates from the PLC and migrates to the plasma membrane (1). Additional backup quality-control mechanisms exist in case suboptimally loaded MHC class I molecules have been released from the ER (16–19). Peptide Ags are eventually presented at the cell surface to cytotoxic CD8+ T cells, which can kill the target cells, if they recognize the peptide as abnormal or of nonself origin (2).

TAP is a member of the ATP-binding cassette (ABC) transporter family (4). The molecule forms a heterodimer consisting of two subunits, TAP1 and TAP2, and resides in the ER membrane where it shuttles peptides from the cytosol into the ER (4). Both TAP subunits have a similar domain structure, in which an N-terminal TMD is followed by a cytosolic C-terminal nucleotide binding domain (Fig. 1A). The nucleotide binding domain selects and hydrolyzes nucleotides to energize the transport cycle (4, 20). The TMD can be further subdivided into an N-terminal domain (N domain), containing four membrane-spanning segments in TAP1 and three membrane-spanning segments in TAP2, and a central core TMD (coreTMD) containing six transmembrane (TM) helices (Fig. 1A) (21). The coreTMD binds the peptide ligands and forms the translocation pore (4). The N domains in both TAP1 and TAP2 are not essential for peptide transport, but each contains one single independent docking site for tapasin (18, 22–24). Hence within the PLC, each assembled TAP1:TAP2 heterodimer interacts with two molecules of tapasin (18, 22, 25, 26). For both of these interactions, tapasin uses its single TM segment (7, 8), which has been reported to associate with the first membrane-spanning helix in the N domain of TAP1 and TAP2 (6). Recently, a third tapasin binding site was described that is located within the coreTMD of TAP1 (18). This binding site, however, appears to be accessible only in unassembled TAP1 chains and not in assembled TAP, indicating that TAP2 and tapasin may compete for binding to an overlapping surface area of the molecule (18). In contrast, no tapasin binding was detected to the coreTMD of TAP2 (18).

Besides the formation of the PLC, the interaction between TAP and tapasin serves another major purpose: to stabilize the transporter. In human cells lacking tapasin, TAP levels may be 3- to 10-fold reduced, whereas studies on tapasin-deficient murine cells reported >100-fold lower TAP levels than in wild-type (wt) cells (7–9, 27, 28). However, it is unknown whether tapasin causes

Abbreviations used in this article: ABC, ATP-binding cassette; coreTMD, core transmembrane domain; ER, endoplasmic reticulum; N domain, N-terminal domain; PLC, peptide loading complex; RT, room temperature; TM, transmembrane; TMD, transmembrane domain; wt, wild-type.

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higher steady-state levels of TAP via binding to the N domain of TAP1, the N domain of TAP2, or the coreTMD of unassembled TAP1 before transporter heterodimerization. In fact, all of these interactions could be necessary to achieve full TAP stability. Moreover, the tapasin docking site in the coreTMD of TAP1 has not been characterized, and it is unclear whether it serves a function in transporter assembly, stability, or both. To address these issues, we performed extensive mutagenesis on the coreTMD of TAP1 and identified the TM segment TM9 as the tapasin binding site in this domain. TM9 forms an amphipathic α-helix, and we find the polar face of this segment to be essential for tapasin association. Within this polar face we identify six critical amino acids that, if mutated, abrogate tapasin binding. Strikingly, despite being functional TAP1 chains, the tapasin binding mutants display poor heterodimerization with TAP2, suggesting that the interaction of tapasin with the coreTMD of TAP1 may be necessary for proper TAP assembly. Moreover, docking of tapasin to both the N domain and the coreTMD of unassembled TAP1 is required for full stability of the subunit: eliminating either binding site alone has only a small effect, but mutating both simultaneously causes a drastic reduction in stability. Further analysis of N-terminal deletion mutants of both TAP1 and TAP2 suggest that all three tapasin binding sites may cooperate to facilitate assembly of the intact transporter.

Materials and Methods

Cell lines and cell culture

T2 is a human lymphoblastoid cell line expressing HLA-A2 and HLA-B51 (29). Untransfected T2 cells or transfectants of T2 expressing wt-TAP1 or TAP1 mutants were cultured in IMDM (Sigma), 10% FCS (HyClone) containing nonessential amino acids (Invitrogen), GlutaMax (Invitrogen), and penicillin/streptomycin (Invitrogen). T2 cells expressing wt-TAP2 or TAP2 mutants were grown in medium additionally containing 2 mg/ml G418 (Life Technologies). T2 cells coexpressing wt-TAP1 and wt-TAP2, ∆N and wt-TAP2, or wt-TAP1 and ∆2N have been described previously (25).

Antibodies

R. SinF (30), R. RingAC (31), and R. gp48N (5) are rabbit polyclonal Abs raised against soluble recombinant tapasin, the C terminus of TAP1, and the N terminus of tapasin, respectively. 36H, a mouse mAb raised against soluble recombinant tapasin, the C terminus of TAP1, and tapasin binding mutants display poor heterodimerization with TAP2 (in pLNCX2) were sequenced in both directions before retroviral transduction into T2 cells were ligated into the XhoI/NotI sites of the retroviral vector pBMN-ires-EGFP as XhoI/NotI fragments. Constructs ∆N-R-Q, ∆N-R-Q, ∆N-N411I, ∆N-N411K, ∆N-N411A, ∆N-T415Q, ∆N-T415A, ∆N-G419A, and ∆N-G419H were generated by QuikChange mutagenesis using ∆N in pBMN-ires-EGFP as template vector in combination with primer pairs R-QF/R-QB, R-QFR/QRB, N411YF/N411YB, N411KE/N411KB, T415QF/T415QB, T415AF/T415AB, G419AF/G419AB, and G419HF/G419HB, respectively. Construct ∆N-K423Q* in pBMN-ires-EGFP was an accidental product generated via QuikChange mutagenesis using earlier identified primer pair R-QF/QRB but contained the additional unwanted mutation M202I. The latter unwanted mutation was removed by swapping sequences between ∆N and ∆N-R-Q. This allows for better resolution of ∆N-K423Q in pBMN-ires-EGFP.

Cells were stained with ∆N-R-QH in pBMN-ires-EGFP as template vector in combination with primer pairs R-HQF/R-HQB and LYLVRHQF/LYLVRHQB. Constructs ∆N-YR-HQ, ∆N-YLVR-HQ, and ∆N-LR-HQ were generated by QuikChange mutagenesis using ∆N-RHQ in pBMN-ires-EGFP as template vector in combination with primer pairs YHRHQF/YHRHQB, YLRHQF/YLRHQB, and YLRHQF/LYRHQB, respectively. Constructs ∆N-3AQ and ∆N-5AQ were generated by QuikChange mutagenesis using ∆N-K423Q in pBMN-ires-EGFP as template vector in combination with primer pairs 3AQF/3ABQ and 5AQF/5ABQ, respectively. Constructs TAP1-3AQ and TAP1-5AQ were generated by ligating the 1296-bp XcmI/XcmI fragment derived from ∆N-3AQ and ∆N-5AQ, respectively, into XcmI-cleaved TAP1 in pBMN-IRES-EGFP, thereby replacing the corresponding 1296-bp segment in full-length TAP1.

Traffic and sorting

All vectors that contain mutant or wt TAP1 (in pBMN-ires-EGFP) or TAP2 (in pLNCX2) were coexpressed in both directions before retroviral transduction into T2 cells (37). A detailed transduction protocol was reported by Leonhardt et al. (38). T2 transfectants expressing TAP1 derivatives were either sorted twice for EGFP expression or cloned by limiting dilution or both to obtain a TAP1 population. T2 transfectants expressing TAP2 derivatives were selected in medium containing 2 mg/ml G418 (Life Technologies) and cloned by limiting dilution.

Immunofluorescence, flow cytometry, and Western blotting

Immunofluorescence microscopy was performed as described previously (39). In brief, T2 transfectants were washed with PBS and fixed with 2% formaldehyde (15 min at room temperature [RT]). After quenching with 1% sodium borohydride, a wash with PBS, and staining with ∆N was permeabilized for 1 h in staining buffer (PBS/0.5% BSA/0.5% saponin) and stained for 1 h with the TAP1-specific Ab 148.3 (1:100) in staining buffer. Ab 148.3 has been used in immunofluorescence applications before (40). After three washes with staining buffer, an Alexa 647-conjugated goat anti-mouse IgG secondary Ab (Molecular Probes) was applied at a 1:100 dilution in the same buffer, before cells were washed with 1% paraformaldehyde. Long gold reagent (Invitrogen), and analyzed by confocal fluorescence microscopy using a Leica TCS SP2 Confocal Microscope (Leica Microsystems).

Flow cytometry was performed as described previously (40), using the Ab 4E directly conjugated to Alexa 647 at a concentration of 1:30. Live-cell gating was performed using YO-PRO-1 iodide (Invitrogen) or propidium iodide (Sigma). All data were acquired on a FACSCalibur flow cytometer (BD Biosciences) and analyzed using FlowJo 6.4.7 software. (Tree Star). Western blotting was carried out as described previously (26). Importantly, because TAP1 and TAP2 are multispanning membrane proteins,
lysates were not boiled to avoid aggregation but vortexed for 30 min at RT for denaturation.

**Pulse-chase analysis and immunoprecipitation**

Radiolabeling was performed as described previously (41). In brief, 2.2 × 10^7 starved T2 cells expressing individual TAP1 subunits or heterodimeric TAP transporter derivatives were pulse labeled at 37°C with [35S]methionine/cysteine (PerkinElmer Life Sciences) at 0.5 mCi/ml in 2.2 ml for 1 h and subsequently chased in IMDM, 10% FCS containing an excess of cold l-methionine/-cysteine (both at 0.45 mg/ml) for up to 8 h. Following this, cells were harvested and frozen at −80°C until the next day or immediately lysed in 1% digitonin (Calbiochem; containing protease inhibitor mixture [Roche Applied Science]) at 10^7 cells/ml and precleared overnight using protein A.

For immunoprecipitation, Ab 148.3-coupled protein A-Sepharose was used. If the preclear had not been performed overnight, the frozen cell pellets were thawed, lysed in 1% digitonin (Calbiochem; containing protease inhibitor mixture [Roche]) at 10^7 cells/ml, and precleared using protein A-Sepharose beads. Subsequently, the supernatant was applied to 148.3-coupled beads and immunoprecipitation was carried out as described previously (19). In brief, supernatants were incubated with Ab-coupled beads for 2 h at 4°C on a rotator and washed five times with PBS/0.1% digitonin before bound proteins were eluted by vortexing in 100 mM Tris/0.5% SDS for 30 min at RT. Because TAP1 and TAP2 are multi-spanning membrane proteins, immunoprecipitates were not boiled in SDS sample buffer to avoid aggregation, but vortexed for 30 min at RT for denaturation. After separation of immunoprecipitates by SDS-PAGE, gels were dried, exposed to PhosphorImager screens, and analyzed with ImageQuant 5.2 (GE Healthcare). Immunoprecipitations from nonradioactively labeled cells were carried out analogously after lysis of 10^7 cells/ml and precleared overnight using protein A.

**RT-PCR**

Total RNA was extracted from T2 transfectants using the RNeasy Mini Kit (Qiagen), and cDNA was prepared using the First Strand Synthesis Kit (Stratagene). Actin-specific primers have been described previously (39). To detect TAP2, we used the primer pair 5'-ACCCTGATGAGTAACTGG-3' and 5'-AACGCCTTTTCACCAATGTTT-3' to amplify a 267-bp fragment in a standard Taq-driven PCR (annealing temperature 60°C/35 cycles).

**Results**

The coreTMD of unassembled human TAP1 contains a tapasin binding site that involves TM segment TM9

Tapasin interacts with both the human and the rat assembled antigenic peptide transporter TAP via the N domains of TAP1 and TAP2 (18, 23, 24). In addition, unassembled rat TAP1 subunits, but not rat TAP2 subunits, can interact with human tapasin via their coreTMD, but this interaction is lost once the transporter chain heterodimerizes with TAP2 (18). The nature of this interaction remains undefined. It is also well established that tapasin substantially stabilizes TAP, allowing significantly higher levels of TAP protein to accumulate in the cell (7–9, 27, 28, 42). However, whether tapasin promotes TAP stability through binding to the N domains of TAP2 and TAP1 or through binding to the coreTMD of unassembled TAP1, or through all of these interactions is unknown. To address these issues, we aimed to construct a mutant TAP1 subunit lacking the tapasin binding site within the coreTMD and analyze its capability to restore TAP function and MHC class I-mediated Ag presentation in TAP-deficient T2 cells.

Because this interaction had so far only been described to occur between rat TAP1 and human tapasin in a hybrid experimental system (18), we first examined whether it could also be observed in a purely human system. To this end, we generated a functional human N-terminal deletion mutant of TAP1 (Δ2-162) lacking the N domain but retaining the coreTMD (25), referred to as 1ΔN (Fig. 1A, 1B). To assess whether this construct can interact with tapasin, we generated stable clones of the human TAP-deficient cell line T2 expressing either full-length wt TAP1 or 1ΔN, and performed an immunoprecipitation from digitonin lysates with either TAP1-specific (148.3) or tapasin-specific (PaSta-1) Abs. As shown in Fig. 1C and 1D, 1ΔN clearly associated with tapasin, although to a lower extent than wt-TAP1.

Tapasin has been reported to use its single TM segment to associate with the first TM helix of both N domains in TAP (6–8). We

![Image 1](http://www.jimmunol.org/)

**FIGURE 1.** The coreTMD of human TAP1 contains a tapasin binding site. (A) Schematic representation and domain organization of the TAP subunits TAP1 (black) and TAP2 (red). (B) Schematic representation of the 1ΔN construct lacking the N domain. (C and D) TAP1 (C) or tapasin (D) were immunoprecipitated with the indicated Abs from digitonin lysates of untransfected T2 cells or stable T2 transfectants expressing full-length wt TAP1 or construct 1ΔN. Isolated proteins were analyzed by Western blotting using Abs against TAP (148.3), tapasin (R.SinE), and MHC class I (HC10). Vertical dashed lines indicate positions where irrelevant lanes have been removed from the image.

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therefore speculated that the interaction between the chaperone and the coreTMD of unassembled TAP1 might also involve TM segments. Recently, the topology of TAP1 and, in particular, the positions of the TM helices within TAP1 had been determined (21). Because previous studies had indicated that only unassembled TAP1 but not unassembled TAP2 associates with tapasin via the coreTMD (18), we exchanged each of the six TM helices (TM5 through TM10) in the coreTMD of 1ΔN individually to the respective sequence counterpart of the collinear TAP2 molecule (Fig. 2A). This strategy gave rise to six different 1ΔN variants, 1ΔN-TM5 through 1ΔN-TM10, in which each one TM segment was replaced by the corresponding TAP2-derived sequence (Fig. 2B). All these mutants were stably expressed in T2 cells and analyzed by Western blotting. 1ΔN-TM9 could be expressed at normal levels when compared with the parental 1ΔN construct, 1ΔN-TM5, at somewhat reduced levels, and the other four constructs (1ΔN-TM6, 1ΔN-TM7, 1ΔN-TM8, and 1ΔN-TM10) only at very low levels (data not shown). To determine whether these mutants were capable of interacting with tapasin, we immunoprecipitated them with the anti-TAP1 mAb 148.3 from digitonin lysates and assessed the coprecipitation of tapasin (Fig. 2C–G). Constructs 1ΔN-TM5 and 1ΔN-TM7, although expressed at reduced levels, efficiently co-isolated tapasin at near-normal levels when normalized to immunoprecipitated 1ΔN (Fig. 2C, 2E), indicating that TM5 and TM7 are not involved in tapasin binding. Construct 1ΔN-TM8 also bound tapasin even though the interaction appeared substantially diminished (Fig. 2F). Constructs 1ΔN-TM6 and 1ΔN-TM10 displayed no tapasin binding at all (Fig. 2D, 2F); but given the very low expression levels of these mutants even in clones selected for highest expression (data not shown), these results may reflect folding issues rather than the loss of a specific interaction. Strikingly, though, construct 1ΔN-TM9, which was expressed at levels equal to or higher than the parental 1ΔN, completely lacked tapasin binding (Fig. 2G). Next, we compared the subcellular distribution of 1ΔN-TM9 and 1ΔN by confocal immunofluorescence microscopy, and for both constructs we observed an identical reticular staining pattern with additional labeling surrounding the nucleus, demonstrating that both mutants properly localized to the ER (Fig. 3A). This indicates that loss of tapasin binding by 1ΔN-TM9 did not reflect a loss of ER retention.

One major advantage of exchanging TM segments between TAP2 and TAP1 rather than using TAP1 deletion constructs is that the respective mutants may retain function, which would indicate that they are properly folded, and hence that loss of tapasin binding is not a result of major structural defects. To assess whether 1ΔN-TM9 is functional, we transduced the respective T2 transfectant with TAP2 to determine whether heterodimerization occurred. Indeed, when 1ΔN-TM9 was immunoprecipitated with TAP1-specific Abs, TAP2 was coisolated, indicating that this mutant subunit was able to associate with TAP2 (Fig. 3B). Moreover, in a functional flow
cytometry assay, monitoring the ability of the 1ΔN-TM9/TAP2 transporter to promote the surface expression of the TAP-dependent molecule HLA-B*5101, we observed an HLA-B,C+ population after TAP2 transduction into cells pre-expressing 1ΔN-TM9 (Fig. 3C, 3D) (this experiment was performed before selection after TAP2 transduction into cells pre-expressing 1ΔN-TM9/1, which was also shown to be dispensable for tapasin binding. However, very likely because of the neutralization of residue Lys 423, construct 1ΔN-TM9/1 (Fig. 4K, lane 4) did not interact with tapasin (Fig. 4D), although it was retained in the ER (Fig. 4E), suggesting that it is the identity of the N terminus of the TAP1 TM9 that is important for tapasin binding.

The TAP2 TM segment TM8 (as given in the Swiss Protein database [21]), which corresponds to TAP1 TM segment TM9 (Fig. 1A), contains a prominent pair of positively charged arginine residues in its N-terminal third (Fig. 4A). Thus, it was tempting to speculate that the introduction of these basic residues disrupted tapasin binding in constructs 1ΔN-TM9, 1ΔN-TM9/1, and 1ΔN-TM9/2. To test this hypothesis, we mutated the respective residues, Thr415 and Ser416, to arginine individually. In addition, we included a construct 1ΔN-R1Q, which suggested that the simultaneous presence of basic residues in the N-terminal and in the middle segments of TM9 may cause poor expression, we neutralized Lys423 by changing it to the glutamine found in TAP2 (Fig. 4F). This strategy allowed the successful expression of high levels of three constructs, containing arginine at position 415 (1ΔN-R1Q), 416 (1ΔN-R1Q), or at neither of the two positions (1ΔN-K423Q, Fig. 4G, 4G). Interestingly, the K423Q exchange alone appeared to result in weaker tapasin binding as shown by slightly reduced coprecipitation of the chaperone when compared with 1ΔN, particularly when levels of coisolated tapasin were normalized to levels of precipitated 1ΔN (Fig. 4H, lane 3). The additional introduction of arginine at position 415 (in 1ΔN-R1Q), but not at position 416 (in 1ΔN-R2Q) reduced tapasin binding (Fig. 4H, lanes 4, 5), indicating that Thr415 and Lys423 contribute to the interaction (Fig. 4J, see red asterisks in helical wheel model of TM9).

Although tapasin association with construct 1ΔN-R1Q was clearly diminished (Fig. 4H), substantial residual binding could still be detected. We therefore aimed to introduce additional mutations abrogating this binding activity. To this end, we generated two constructs, which were both based on the mutant 1ΔN-R1Q. In the first, all TM9 residues N-terminal to Thr415 (residues 410-414) were exchanged to their counterparts in TAP2, giving rise to construct 1ΔN-LYLLVRQ (Fig. 4I). This construct represents a variant of the poorly expressed 1ΔN-TM9/1 not containing the mutation at residue 416, which our results in Fig. 4H had already shown to be dispensable for tapasin binding. However, very likely because of the neutralization of residue Lys423, construct 1ΔN-LYLLVRQ could be expressed at much higher levels than 1ΔN-TM9/1 (Fig. 4K, lane 4). Interestingly, construct 1ΔN-LYLLVRQ displayed a drastic reduction in tapasin binding when compared with the parental construct 1ΔN-R1Q (Fig. 4L, lanes 3, 4), suggesting that the first N-terminal third of the TM9 plays a key role in the interaction. Furthermore, we generated a second mutant, 1ΔN-RHQ, also based on construct 1ΔN-R1Q, in which we additionally exchanged Gly410 to the histidine found in TAP2 (Fig. 4I). Gly410 was chosen because it lay between critical residues Thr415 and Lys423 in both the primary amino acid sequence (Fig. 4I) and

FIGURE 3. 1ΔN-TM9 localizes to the ER and retains function to a significant extent. (A) Immunofluorescence analysis of the indicated T2 transfectants using the TAP1-specific Ab 148.3. Original magnification ×63. (B) TAP1 was immunoprecipitated with Ab 148.3 from a digitonin lysate of the stable T2 transfectant coexpressing 1ΔN-TM9 and TAP2. Isolated proteins were analyzed by Western blotting using Abs against TAP1 (148.3) and TAP2 (435.3). (C and D) Flow cytometry analysis of surface HLA-B,C expression using Ab 4E in the indicated T2 transfectants (C). Surface HLA-B,C levels are shown as a bar diagram (1ΔN; TAP2 set to 100%) (D).
the helical wheel projection of TM9 (Fig. 4J). 1ΔN-RHQ was also expressed well (Fig. 4K), and the G419H mutation did substantially affect tapasin association (Fig. 4L, lanes 3, 5), suggesting a possible role for Gly 419 in the binding.

Next, we focused on the role of residues in the first N-terminal third of TM9. We started with construct 1ΔN-RHQ and additionally exchanged either Asn 411 or Trp 413, or both, to their corresponding residues in TAP2 (tyrosine and leucine, respectively; Fig. 5A). All these mutants were well expressed (Fig. 5B), and the additional N411Y mutation almost completely abrogated tapasin binding (Fig. 5C, see 1ΔN-YRHQ and 1ΔN-YLRHQ1). In contrast, mutation of the highly conserved Try 413 did not affect the tapasin interaction at all (Fig. 5C, compare lanes 4 and 6). Taken together, the combined alteration in construct 1ΔN-YRHQ of 4 aa in the TAP1 TM segment TM9 (N411Y, T415R, G419H, and K423Q) almost eliminated tapasin association.

Finally, in an attempt to totally eliminate tapasin binding, we additionally exchanged residues Ser 412 or Thr 414 in 1ΔN-YLRHQ, or both, to their corresponding residues in TAP2 (leucine and valine, respectively; Fig. 5D, 5E). Individually, these exchanges had only little impact on tapasin binding, but the combined exchange in construct 1ΔN-YLVRHQ completely abrogated any residual interaction (Fig. 5F, lane 7). When projected onto a helical wheel, the N-terminal two thirds of the TAP1 TM segment TM9 display

**FIGURE 4.** The N-terminal third of TM segment TM9 plays an important role in tapasin interaction. (A, F, I) Alignment of the TAP1-TM9, the corresponding TAP2 sequence, and selected mutants. TAP1 sequences are shown in gray. TAP2 sequences are shown in red. (B) Schematic representation of 1ΔN mutant constructs. (C, G, and K) Western blot analysis showing digitonin lysates from stable T2 transfectants using Abs recognizing TAP1 (148.3), Grp94 (9G10), and tapasin (R.gp48N). (D, H, and L) TAP1 was immunoprecipitated with Ab 148.3 from digitonin lysates of untransfected T2 cells or the indicated stable T2 transfectants. Isolated proteins were analyzed by Western blotting using Abs against TAP1 (148.3) and tapasin (R.gp48N). (E) Immunofluorescence analysis of the indicated T2 transfectants using the TAP1-specific Ab 148.3. Original magnification ×63. (J) Helical wheel projection of the first two thirds of the TAP1 TM segment TM9. Residues whose mutation results in reduced tapasin binding are indicated with a red asterisk.
pronounced amphipathicity with hydrophobic amino acids on one face, and mostly polar and one charged amino acid on the other face (Fig. 5I). In contrast, the corresponding TM segment in TAP2 is less amphipathic and contains overall more hydrophobic residues, which are distributed more homogenously all around the helix (Fig. 5G). Strikingly, all residues in the TAP1 TM9 involved in tapasin binding (Asn 411, Ser 412, Thr 414, Thr 415, Gly 419, and Lys423) are located on the polar face of the amphipathic α-helix (Fig. 5I, red asterisks). The respective mutations in construct 1D_N-YLVRHQ largely introduce hydrophobicity in that area, resulting in the loss of tapasin association (Fig. 5G–I). Importantly, the combined results in Figs. 4 and 5 show that the progressive weakening of the polar character of the TM9 leads to a progressive loss of tapasin binding. Thus, we conclude that the amphipathic TM segment TM9 of the coreTMD of TAP1 binds tapasin via its polar face.

Tapasin binding mutant 1ΔN-YLVRHQUERY is not a functional TAP1 chain and displays reduced TAP2 association

Because 1ΔN-TM9 had retained significant functional activity (Fig. 3C, 3D), we expected 1ΔN-YLVRHQ to be a functional TAP1 chain as well. However, when T2 cells pre-expressing this construct were additionally transfected with TAP2 to allow for the formation of assembled TAP heterodimers, no HLA-B,C could be detected at the cell surface (Fig. 6A, 6B), indicating that 1ΔN-YLVRHQ was nonfunctional. To show that TAP2 was successfully expressed in these cells, we performed a TAP2-specific RT-PCR, which confirmed that TAP2 mRNA was made (Fig. 6C). Moreover, lysates from two independent clones of T2 cells coexpressing 1ΔN-YLVRHQ and TAP2 clearly contained TAP2 protein (Fig. 6D), and both of these clones were surface HLA-B,C⁺ (data not shown). However, TAP2 levels were reduced when compared with wt TAP when normalized to an irrelevant ER protein, Grp94 (Fig. 6D, 6E, blue bars), and the reduction was even more pronounced when normalized to TAP1 (Fig. 6D, 6E, black bars). This is important, because human TAP2 is completely unstable without TAP1 and cannot be detected by Western blot at all if expressed in isolation (43, 44) (Fig. 6D, lane 4). Hence every TAP2 molecule that can be detected in Western blot is likely a molecule that has been stabilized by TAP1 in an assembled heterodimer. Consistent with this, TAP2 could indeed be coimmunoprecipitated with the 1ΔN-YLVRHQ subunit (Fig. 6F). We therefore conclude that heterodimerization of 1ΔN-YLVRHQ with TAP2 is impaired but not eliminated (Fig. 6D, 6E). This impairment alone, however, is unlikely to explain the complete absence of surface HLA-B,C expression by T2 cells coexpressing TAP2 and 1ΔN-YLVRHQ, because sufficient levels of heterodimer should exist in the cell to promote at least some surface expression if the mutant transporter were active.

Functional consequences of individual amino acid exchanges in TM segment TM9

To determine which of the six mutations introduced into 1ΔN-YLVRHQ were causing the block in TAP function, we coexpressed a subset of earlier described mutants, 1ΔN-K423Q*, 1ΔN-R1Q, 1ΔN-YRHQ, and 1ΔN-YLVRHQ, containing one, two, four, and six mutations in the TM segment, respectively (Fig. 6G), with TAP2, and analyzed whether the respective transporters could promote HLA-B,C expression by T2 cells coexpressing TAP2 and 1ΔN-YLVRHQ, because sufficient levels of heterodimer should exist in the cell to promote at least some surface expression if the mutant transporter were active.
to affect TAP function significantly (Fig. 6H). Notably, the related homodimeric peptide transporter TAPL/ABCB9 also contains glutamine in this position. However, the additional exchange of Thr415 to arginine in construct 1D\textsuperscript{N-R1Q} substantially diminished TAP activity in this assay, and the further replacement of Asn 411 and Gly419 by tyrosine and histidine, respectively, in construct 1D\textsuperscript{N-YRHQ} resulted in complete loss of function (Fig. 6H). It is not surprising then that 1D\textsuperscript{N-YLVRHQ}, which contains two more mutations (Figs. 6G, 7A), was also inactive (Fig. 6H).

To convert these constructs into functional TAP subunits (yet lacking tapasin binding to the coreTMD), we next assessed the functional significance of all four residues affected in loss-of-function construct 1D\textsuperscript{N-YRHQ} individually. To this end, we started with construct 1D\textsuperscript{N} and mutated Asn 411 to either tyrosine (as in TAP2 and 1D\textsuperscript{N-YRHQ}), lysine, or alanine; Thr 415 was mutated to glutamine or alanine; Gly 419 was mutated to histidine (as in TAP2 and 1D\textsuperscript{N-YRHQ}) or alanine; Lys 423 was mutated to glutamine (Fig. 7B). After transduction of the respective constructs into a T2 cell clone pre-expressing TAP2, we measured surface HLA-B,C levels by flow cytometry as an indicator of TAP activity. Because the 1D\textsuperscript{N} derivatives were linked to EGFP via an IRES site, we gated on EGFP\textsuperscript{+} cells to analyze only cells that had been successfully transfected (Fig. 7C). As expected, cells expressing only TAP2 alone displayed no TAP activity, whereas 1D\textsuperscript{N}, if coexpressed with TAP2, promoted high surface HLA-B,C levels (Fig. 7D, 7E, white histograms). Of the four mutations present in the nonfunctional quadruple mutant 1D\textsuperscript{N-YRHQ}, both N411Y and G419H appeared to dramatically reduce TAP function by \(\sim 75\%\) (Fig. 7E, red bars). If it is taken into account that the Thr 415-to-arginine exchange in 1D\textsuperscript{N-YRHQ} also diminished the activity of the subunit (Fig. 6H), it is not surprising that this construct is nonfunctional. Some of the other mutations we introduced into 1D\textsuperscript{N} also impaired transporter activity. In particular, N411K and T415Q proved to be problematic, causing a significant and moderate reduction in activity, respectively (Fig. 7E, red bars). In contrast, 1D\textsuperscript{N-G419A} transfectants had higher HLA-B,C surface levels than 1D\textsuperscript{N} transfectants (Fig. 7E, red bars), indicating that Gly 419 could be exchanged to alanine without a significant loss of function. Similarly, and consistent with our results in Fig. 6H, the Lys\textsuperscript{223}-to-glutamine exchange also

FIGURE 6. Constructs 1D\textsuperscript{N-YRHQ} and 1D\textsuperscript{N-YLVRHQ} are nonfunctional. (A and B) Flow cytometry analysis of surface HLA-B,C expression using Ab 4E in the indicated T2 transfectants (A). Black histograms correspond to untransfected T2 cells or T2 cells expressing TAP1 mutants alone. Red histograms correspond to T2 cells expressing TAP2 or coexpressing TAP2 and a TAP1 mutant. Surface HLA-B,C levels are shown as a bar diagram (color code as in A) (B). (C) RT-PCR using actin- or TAP2-specific primers. (D and E) Western blot analysis showing digitonin lysates from stable T2 transfectants using Abs recognizing TAP1 (R.RING4C), Grp94 (9G10), and TAP2 (K0137-3). The bar diagram in (E) displays the quantification of the Western blot in (D). TAP2 levels normalized to TAP1 are shown in black. TAP2 levels normalized to Grp94 are shown in blue. (F) TAP1 was immunoprecipitated with Ab 148.3 from digitonin lysates of T2 cells stably coexpressing 1D\textsuperscript{N-YLVRHQ} and TAP2. Isolated proteins were analyzed by Western blotting using Abs against TAP1 (R.RING4C) and TAP2 (K0137-3). (G) Alignment of the TAP1-TM9, the corresponding TAP2 sequence, and selected mutants. TAP1 sequences are shown in gray. TAP2 sequences are shown in red. (H) Flow cytometry analysis of surface HLA-B,C expression using Ab 4E in the indicated T2 transfectants (color code as in A, B). Surface-HLA-B,C levels are shown as a bar diagram.
led to higher HLA-B,C surface levels (Fig. 7E, red bars), suggesting that Lys$^{423}$ is not essential for transporter activity either. Further, the Thr$^{412}$-to-alanine mutant promoted HLA-B,C surface expression to the same extent as the parental construct 1ΔN.

FIGURE 7. Functional analysis of 1ΔN point mutants. (A) Helical wheel projection of the first two thirds of the TAP1 TM segment TM9. Residues whose mutation result in reduced tapasin binding are indicated with a red asterisk. (B) Alignment of the TAP1-TM9 and selected point mutants. TAP1 sequences are shown in gray. Point mutations are highlighted in color. (C) Flow cytometry analysis showing EGFP expression versus forward scatter for the T2 transfectant coexpressing 1ΔN and TAP2. The EGFP$^+$ population is shown in the gate. (D) and (E) Flow cytometry analysis of surface HLA-B,C expression using Ab 4E in the indicated T2 transfectants. Results were first gated on living cells (propidium iodide$^-$), then on EGFP$^+$ cells using the gate shown in (C). Cells expressing TAP2 alone (first histogram in upper row) were not gated on EGFP. The color code of the histograms corresponds to the color code used in (B). The flow cytometry results are shown as a bar diagram in (E) (red bars, surface HLA-B,C levels; green bars, EGFP fluorescence). (F) Alignment of the TAP1-TM9, the corresponding TAP2 sequence, and selected mutants. TAP1 sequences are shown in gray. TAP2 sequences are shown in red. The color code for mutations in constructs 1ΔN-3AQ and 1ΔN-5AQ is adapted from (B), (D), (G), and (H). Helical wheel projection of the first two thirds of the TM segment TM9 in mutants 1ΔN-3AQ and 1ΔN-5AQ. (I and K) Western blot analysis showing digitonin lysates from stable T2 transfectants using Abs recognizing TAP1 (R.RING4C), Grp94 (9G10), or tapasin (R.gp48N). (J and L) TAP1 was immunoprecipitated with Ab 148.3 from digitonin lysates of untransfected T2 cells or the indicated stable T2 transfectants. Isolated proteins were analyzed by Western blotting using Abs against TAP1 (R.RING4C) and tapasin (R.gp48N or 3H6).
(Fig. 7E, red bars). Mutant N411A was about one third less efficient than 1ΔN in promoting HLA-B,C surface levels, a reduction that may be considered moderate (Fig. 7E, red bars).

**Mutations in TM segment TM9 can suppress expression problems of 1ΔN**

In the experiment shown in Fig. 7C–E, we had used a TAP2-expressing clone and transfected it with TAP1 mutants linked to an IRES-EGFP unit. Thus, TAP1 and EGFP should be expressed together on the same mRNA, and because we gated on EGFP+ cells (Fig. 7C), we expected 100% of the analyzed cells to express both the TAP1 mutant and wt TAP2. Surprisingly, though, HLA-B,C surface expression was detected in only ~25% of the 1ΔN-transfected cells, indicating a high rate of uncoupling of TAP1 expression from the EGFP marker (Fig. 7D, note the two peaks in white histogram). This uncoupling was reproducible and always seen when construct 1ΔN was transfected into cells pre-expressing TAP2 (see also Supplemental Fig. 1A, 1B, note the two peaks in white histogram). However, the phenomenon did not depend on TAP2 expression as EGFP+, 1ΔN+ clones were also obtained when 1ΔN alone was transduced into plain T2 cells (data not shown). Interestingly, though, despite the majority of transfectants losing 1ΔN expression after initial introduction of the gene, the population that retained 1ΔN expression stably continued the production of high levels of the mutant indefinitely (data not shown). This suggests that although the sudden introduction of 1ΔN may cause problems in the transfected cells, about a quarter of the transfectants are able to adapt to long-term 1ΔN expression eventually. The same uncoupling phenomenon was also observed in all Asn411 and Thr415 mutants (Fig. 7D, note the two peaks in yellow and blue histograms).

Strikingly, exchanging Gly419 for histidine or alanine resulted in all EGFP+ transfectants expressing the transporter (Fig. 7D, note only one single peak in green histograms). Identical observations were made with the mutant in which Lys423 was exchanged for glutamine, and again, this was reproducible (Fig. 7D, Supplemental Fig. 1A, 1B, note only one single peak in the pink histogram). This may indicate that both Gly419 and Lys423 cause problems for the proper insertion of TM segment TM9 in the wt molecule, and removing those residues relaxes these issues. Consistent with this idea, mutants in which Lys423 was exchanged for glutamine often displayed higher steady-state protein levels than construct 1ΔN (Figs. 4C, 4G, 4K, 5B), although expression levels varied from experiment to experiment. Moreover, EGFP fluorescence in the bulk transfectants shown in Fig. 7D was significantly higher for the Gly419 and Lys423 mutants than for any other construct (Fig. 7E, green bars), further supporting the idea that these mutants were expressed at substantially higher levels.

**Construction of a tapasin binding–deficient TAP1 mutant predicted to retain functional activity**

With the goal of generating a functional version of construct 1ΔN-YRHQ, in which the same residues are mutated but in which the TAP activity was only mildly or not at all affected, we generated a quadruple mutant of 1ΔN containing the amino acid exchanges N411A, T415A, G419A, and K423Q, and named it 1ΔN-3AQ (Fig. 7F). We also constructed a potentially improved variant of the nonfunctional mutant 1ΔN-5AQ, which was based on 1ΔN-3AQ as a parental construct and additionally contained mutations S412A and T414A. This construct was named 1ΔN-5AQ (Fig. 7F). Helical wheel projections show that both 1ΔN-3AQ and 1ΔN-5AQ have lost a charge and contain several hydrophobic amino acids on the formerly polar face of the amphipathic helix TM9 (compare Fig. 7G, 7H, and Fig. 7A). These mutants could be stably expressed in T2 cells (Fig. 7I) and behaved identically to their parental derivatives with respect to tapasin binding. In particular, 1ΔN-3AQ, just as 1ΔN-YRHQ (Fig. 5F), almost completely lost the tapasin interaction, the minimal residual binding requiring long exposure of the respective Western blots to be visualized (Fig. 7J, lanes 3, 5). Further, 1ΔN-5AQ, just as 1ΔN-YLVRHQ (Fig. 5F), displayed no tapasin binding at all (Fig. 7J, lanes 4, 6). Next, we inserted the 3AQ mutation (N411T/G415S/G419V/K423Q→A/A/A/A; Fig. 7F) into full-length TAP1 (instead of 1ΔN) and expressed this derivative in T2 cells (Fig. 7K). TAP1-3AQ clearly displayed strong tapasin binding, suggesting that the interaction with the coreTMD is not essential to allow the chaperone to interact with the TAP1 N domain (Fig. 7L).

TAP1-3AQ and TAP1-5AQ mutants retain functional activity but display reduced heterodimerization with TAP2

Next, we assessed whether the 3AQ and 5AQ mutations affected TAP1 activity. To this end, we first coexpressed in T2 cells the respective 1ΔN derivatives with TAP2 and examined HLA-B,C surface levels (Fig. 8A, 8B). In this assay, both 1ΔN-3AQ and 1ΔN-5AQ were functional TAP1 subunits, although 1ΔN-5AQ displayed reduced activity (Fig. 8A, 8B). Following this, TAP heterodimerization was assessed by Western blotting. Again, human TAP2 alone was undetectable at steady-state (Fig. 8C, lane 2). Interestingly, 1ΔN stabilized significantly less TAP2 than full-length TAP1, and the same was true for 1ΔN-3AQ and 1ΔN-5AQ (Fig. 8C, 8D). We note that this was reminiscent of the differential capability of wt-TAP1 and construct 1ΔN-YLVRHQ to stabilize TAP2 (Fig. 6D, 6E). Moreover, similar observations were also made in radiolabeling experiments (see later in Fig. 9H). We confirmed that different steady-state TAP2 protein levels in the respective cell lines were not caused by disparate TAP2 mRNA expression (Fig. 8E). This suggests that the N domain of TAP1 plays an important role in the heterodimerization of the transporter, which raised the question whether the other two tapasin binding sites, the N domain of TAP2 and the TM segment TM9 in TAP1, are also relevant for proper TAP assembly. To test this, we coexpressed in T2 cells TAP2 with full-length wt-TAP1 or TAP1 containing the 3AQ or 5AQ mutations. In addition, we used a cell line published earlier (25), which coexpressed full-length wt-TAP1 with TAP2 lacking the N domain (2ΔN). All these transporters promoted high levels of surface HLA-B,C and only small, if any, differences to wt TAP were observed (Fig. 8F, 8G). Interestingly, when transporter heterodimerization was assessed in the cell lines, the lack of the N domain in TAP2 seemed to cause a reduction in steady-state TAP2 protein levels (Fig. 8H, 8I, compare lanes 2, 3, 5). Similarly, both TAP1-3AQ and TAP1-5AQ stabilized less TAP2 than wt-TAP (Fig. 8H, 8I, compare lanes 2, 3, 4). Again, all these results were also confirmed in radiolabeling experiments (see later in Fig. 9K) and were not caused by disparate mRNA expression levels in the respective cell lines (Fig. 8I). This indicates that all three regions in TAP, which contain tapasin binding sites (the N domain in TAP1, the N domain in TAP2, and the TM9 in TAP1), are necessary for proper heterodimerization and full stabilization of TAP2. The effect of the coreTMD in TAP1, however, may depend on tapasin binding to the N domain in the same subunit, as constructs 1ΔN-3AQ and 1ΔN-5AQ showed only little, if any, reduction in TAP2 stabilization when compared with construct 1ΔN (Fig. 8C, 8D).

Tapasin binding mutants are less stable when unassembled and form less stable TAP heterodimers

A major role of tapasin in the MHC class I Ag presentation pathway is the stabilization of TAP (7–9, 27, 28, 42), but it is unknown
which tapasin binding site on the transporter subunits is required for this function or whether all are required. To begin to address this question, we performed pulse-chase experiments in which we analyzed the stability of unassembled mutant or wt TAP1 chains stably expressed in T2 cells (Fig. 9A–E). Because of the generally very high stability of both assembled and unassembled TAP1 (45), we chased cells for up to 8 h after labeling. As expected, wt-TAP1 did not noticeably decay at all during this time period (Fig. 9A–E, black lines). In contrast, both 1ΔN and TAP1-3AQ, lacking tapasin binding to the N domain and the coreTMD of TAP1, respectively, displayed a slightly diminished stability (Fig. 9C–E, green and red lines). However, the effects were rather small, and a large number of independent experiments were necessary to show that the difference is statistically significant at the 8-h time point. It seems unlikely that this difference could cause the 3- to 10-fold reduction in TAP1 levels that have been observed in the absence of tapasin (7, 8), let alone the 100-fold reduction in TAP levels that has been observed in tapasin-deficient mouse cells (27). Strikingly, however, constructs simultaneously lacking tapasin binding to both the N domain and the coreTMD (1ΔN-3AQ and 1ΔN-5AQ) dramatically lost stability and presented with half-lives of only 4 h (Fig. 9A, 9B, green and blue lines). These results suggest that tapasin binding to both binding sites in TAP1 is necessary to achieve the full stability of unassembled TAP1 chains.

FIGURE 8. Tapasin binding mutants of TAP retain peptide transport activity but display impaired heterodimerization. (A and B) Flow cytometry analysis of surface-HLA-B,C expression using Ab 4E in the indicated T2 transfectants. Results were gated on living, propidium iodide− cells (A). Flow cytometry results showing the averages of four independent experiments including the one depicted in Fig. 8A are shown as a bar diagram (B). Error bars are showing the SD from the mean. Statistical analysis was performed by a repeated-measures ANOVA test followed by Dunnett’s posttest using Prism 4.0 (GraphPad Software). (C and D) Western blot analysis showing digitonin lysates from stable T2 transfectants using Abs recognizing TAP1 (R.RING4C), Grp94 (9G10), and TAP2 (K0137-3) (C). Band intensities were determined densitometrically and are expressed as TAP2:TAP1 ratios in a bar diagram (D). (E) RT-PCR using actin- or TAP2-specific primers. (F and G) Flow cytometry analysis of surface-HLA-B,C expression using Ab 4E in the indicated T2 transfectants. Results were gated on living, propidium iodide− cells (F). Flow cytometry results showing the averages of three independent experiments including the one depicted in (F) are shown as a bar diagram (G). The error bars are showing the SD from the mean. Statistical analysis was performed by a repeated-measures ANOVA test followed by Dunnett’s posttest using Prism 4.0 (GraphPad Software). (H and I) Western blot analysis showing digitonin lysates from stable T2 transfectants using Abs recognizing TAP1 (R.RING4C), Grp94 (9G10), and TAP2 (K0137-3) (H). Band intensities were determined densitometrically and are expressed as TAP2:TAP1 ratios in a bar diagram (I). (J) RT-PCR using actin- or TAP2-specific primers.
Next, we analyzed the effect of the tapasin binding mutations on the stability of assembled TAP heterodimers (Fig. 9F–K). All TAP1 chains lacking the N domain, even 1ΔN, which is fairly stable when unassembled (Fig. 9A–C, 9E, red lines), displayed a dramatic loss of stability when coexpressed with TAP2 (Fig. 9F, 9G, left panel), perhaps because TAP2 obscures the tapasin binding site in the coreTMD of 1ΔN (18, 23, 24). TAP2 also decayed faster when associated with these TAP1 mutants (Fig. 9F, 9G, right panel), although the accelerated degradation was less pronounced. Strikingly, full-length TAP1 mutants containing the 3AQ or 5AQ mutation were also unstable when coexpressed with TAP2 (Fig. 9I, 9J, left panel) and also conferred

**FIGURE 9.** Assembled and unassembled TAP requires tapasin binding for full stability. (A–J) Pulse-chase experiments assessing the stability of unassembled TAP1 single chains (A–E) and assembled TAP (F–J). The indicated T2 transfectants were pulse-labeled for 1 h with [35S] and subsequently chased for up to 8 h. Digitonin lysates derived from the respective cells were immunoprecipitated with the TAP1-specific Ab 148.3, eluted by vortexing in 0.5% SDS at RT, and analyzed by autoradiography (A, C, F, I). Quantitative PhosphorImager analysis of the pulse-chase data (0-h time point set to 100%) is shown (B, D, F, G, J). (B, D, E and G, J, left panels) TAP1 is displayed. (G and J, right panels) TAP2 is displayed. Error bars in all panels are showing the SEM. (B) Displays the averages of three independent experiments including the one depicted in (A). (D) Displays the averages of five independent experiments including the one depicted in (C). (E) Displays the averages of seven independent experiments including the one depicted in (C). Data of the seven experiments depicted in (E) include the three experiments shown in (B). (G) Displays the averages of three independent experiments including the one depicted in (F). (J) Displays the averages of three independent experiments including the one depicted in (I). Statistical analysis using Prism 4.0 (GraphPad Software) was carried out on the values from each time point, normalized in a way that the 0-h time point is set to 100%. For (B), statistical analysis was performed by a repeated-measures ANOVA test followed by Dunnett’s posttest (comparing results against 1ΔN; *p < 0.05, **p < 0.01). For (D), statistical analysis was performed by a two-tailed paired t test (*p = 0.0370, **p = 0.0024). For (E), statistical analysis was performed by a two-tailed paired t test (*p = 0.0329). For (G) and (J), statistical analysis was performed by a repeated-measures ANOVA test followed by Dunnett’s posttest (comparing results against TAP1; TAP2; *p < 0.05, ***p < 0.01). (H and K) Nonnormalized TAP2:TAP1 ratio at time point 0 h for the experiments depicted in (F), (G), (I), and (J), respectively.
less stability on TAP2 (Fig. 9I, 9J, right panel). This suggests that binding of tapasin to the coreTMD of TAP1 may not only be required to generate a normal quantity of TAP heterodimers (Figs. 8I, 8L, 9I, 9K), but may also regulate their ultimate stability.

Discussion
MHC class I–mediated Ag presentation is critical for an appropriate immune response against tumors and viruses (1, 2). The pathway depends on the supply of ER-localized MHC class I molecules with peptides, which are mostly of cytosolic origin, the loading of these peptides into the peptide binding groove of MHC class I, and their subsequent display at the cell surface to cytotoxic CD8+ T cells (1, 2). TAP is a key player in this program, mediating the transport of the cytosolic peptides into the ER (4). In the absence of TAP, MHC class I molecules are starved of peptides and, as a consequence, their surface levels collapse, causing T cells not to recognize their targets anymore (46). Hence it is not surprising that to evade a proper immune response, many tumors downregulate or even completely eliminate TAP expression (2, 46), whereas viruses frequently encode specific TAP inhibitors, such as US6, ICP47, or UL49.5 (47).

To address these questions, we first mapped and characterized the tapasin binding site in the coreTMD of unassembled TAP1. Four amino acid residues in the TM segment TM9 appear to be particularly important: Asn411, Thr413, Gly419, and Lys423. If these residues are mutated to either the corresponding residues in TAP2 (see construct 1D, 1B), the corresponding residue in TAP2 and in the TAP-related ABC transporter ABCC10 (Supplemental Fig. 2A–C) and one based on the outward-facing conformation of SAV2866 (Supplemental Fig. 2D–F), in an evaluation of the potential orientation of these critical residues within TM9. Interestingly, in both models, none of the four amino acids appears to be accessible from the outside of the assembled transporter. Rather, they point either sideways toward TM helix TM8 or into the pore of the transporter (green residues in Supplemental Fig. 2A–F). This interpretation of the arrangement is compatible with the fact that the interaction between tapasin and the coreTMD of TAP1 is not observed in the assembled TAP1:TAP2 heterodimer (18, 23, 24). Moreover, it would suggest that in unassembled TAP1, the TM9 segment is rotated outward within the plane of the membrane so that the key residues become accessible to the docking tapasin molecule.

Much is yet to be learned about interactions between TM helices within lipid bilayers, but a few particular sequence motifs used for such interactions have been identified, including GxxxG (where G stands for glycine and x stands for any amino acid) and its more degenerate cousin SmxxxSm (where Sm stands for a small amino acid, such as glycine, alanine, serine, or threonine) (49). Two of the residues in the TM segment TM9 that we identified as critical for tapasin binding, Thr413 and Gly419, participate in such an SmxxxSm motif (TxxxG; Fig. 2A) and, interestingly, human tapasin may contain a corresponding SmxxxSm motif (GxxxA) spanning residues Gly405 through Ala409 (405-GLFKA-409). Besides specific sequence motifs, small polar residues, such as serine, threonine, and asparagine, have often been found to contribute to interactions between TM helices (50). Of the four residues mutated in construct TAP1-3AQ, Asn411 and Thr413 are polar, and both residues that are additionally mutated in construct TAP1-5AQ, Ser412 and Thr414, are polar as well (Fig. 7F). Together with Lys423, all these residues form an extensively polar face in the amphipathic helix TM9 (Fig. 7A). Notably, the stepwise weakening of this polar character causes a progressive loss of tapasin binding, which is completely eliminated in construct 1ΔN-5AQ, in which the polar face has been essentially destroyed (Figs. 4L–L, 5A–F, 7F–J).

Thus, consistent with the view that polar residues can make important contributions to interactions between TM helices (49, 50), we propose that the polar face of the TM9 segment mediates the association of unassembled TAP1 with tapasin. We note that although the single TM helix of human tapasin is not exceptionally amphipathic, it does contain a conserved lysine residue at position 408, which lies within the earlier mentioned GxxxA motif, and which has been shown to be critical for TAP stabilization (42).

The TM segment TM9 is not well predicted by topology prediction algorithms (21), probably because of its relatively low hydrophobicity. However, direct topology analysis demonstrated that Thr413 is located inside the cytosol, whereas Ser412 is located inside the ER, showing that the sequence between these two residues, which flank TM9, must span the ER membrane in a cytosol-to-ER orientation (21). Various aspects of our analysis suggest that this segment may cause problems for the membrane integration of TAP1 or at least for the membrane integration of its functional (25) derivative 1ΔN. In particular, a subset of T2 cells forced to produce 1ΔN (and many of its derivatives) by transduction appears to have a strong tendency to lose expression of the construct even though they retain EGFP, expressed from the same mRNA via an IRES site (Fig. 7C, 7D, Supplemental Fig. 1A, 1B). The mechanism behind this uncoupling and why it occurs only in some but not in all cells (see Results) is unclear. Strikingly, this problem can be completely eliminated by mutation of the TM segment TM9. One way to fully suppress the TAP1/EGFP uncoupling is to replace the charged Lys423 with glutamine (Fig. 7C, 7D, Supplemental Fig. 1A, 1B), the corresponding residue in TAP2 and in the TAP-related peptide ABC transporter TAP1/ABCB9. This not only reproducibly abrogates the loss of 1ΔN by a subset of cells, but also appears to allow overall higher expression of the mRNA as both EGFP levels (Fig. 7E, Supplemental Fig. 1A) and 1ΔN protein levels are increased (Fig. 4G).

The problem of 1ΔN expression is also resolved by the exchange of Gly419 with either alanine or histidine (Fig. 7C–E). Although it may be obvious that a charged amino acid such as Lys423 might be problematic in a TM segment, it is less clear why glycine would cause such difficulties. Although regarded as a classical “helix breaker” in soluble proteins, glycine is a very common amino acid in TM helices, and the hydrophobic environment within the membrane bilayer typically favors its incorporation into such segments (50). Nevertheless, constraining the glycine backbone by tethering it into a helical structure has an entropic cost (50) and may make Gly419 in its particular environment a suboptimal amino acid in that position. Given that both
Lys423 and Gly419 appear to be involved in tapasin binding, it is possible that it is not the nature of the TM segment as such but the tapasin interaction with the coreTMD that is ultimately causing the expression problems of 1ΔN. However, arguing against this, the 1ΔN-K423Q mutant largely retains tapasin binding activity (Fig. 4H), and even mutant 1ΔN-RHQ, in which both Gly419 and Lys423 are mutated, still displays a low level of tapasin association (Fig. 4L, 5C). Nevertheless, we cannot exclude the possibility that tapasin binds to these mutants in a subtly modified manner compared with the parental 1ΔN construct.

Nothing is known so far about a possible function that tapasin binding to the coreTMD of unassembled TAP1 may serve. Thus, the mutants generated in this study could also provide some functional insights into this interaction. To this end, we had constructed all our mutants as domain-swapping or point mutants rather than deletion mutants to produce potentially functional transporter subunits whose activity, and thus overall proper folding, can be confirmed in assays such as flow cytometry analysis of HLA-B,C cell-surface levels (Fig. 8A, 8B, 8F, 8G). Interestingly, TAP1 lacking the tapasin interaction with the coreTMD appears to be less efficient in forming a dimer with TAP2 (Figs. 8H–J, 9K), and a similar defect was observed with both TAP1 and TAP2 N domain deletion mutants (Figs. 8C–E, 8H–J, 9H, 9K). Consistent with this, the association of rat-derived 1ΔN and 2ΔN was previously shown to be strongly diminished in vitro compared with the intact subunits (51). This suggests that all three tapasin binding sites in the TAP subunits may cooperate to achieve maximally efficient dimer formation.

Interestingly, the absence of the tapasin binding site in the coreTMD appears to have a significant negative effect on heterodimerization only if tapasin binding to the N domain of TAP1 is intact; that is, introducing the 3AQ or 5AQ mutation affects the heterodimerization of full-length TAP1 (Figs. 8H–J, 9K) but does not further impede dimer assembly when introduced into 1ΔN (Figs. 8C–E, 9H). One possible explanation for this could be that the two tapasin binding sites in unassembled TAP1 cooperate in tethering and orienting a shared tapasin molecule in a manner that allows efficient association with an incoming TAP2 molecule. In such a model, disturbing either tapasin docking site could be sufficient to altogether lose the assistance the chaperone normally provides to the process. To form a complete TAP dimer, TAP1 must be made first and subsequently associate with a newly synthesized, nascent TAP2 chain (45). TAP2 entering the complex, however, would have to displace tapasin from the coreTMD because assembled TAP cannot sustain this interaction (18). The chaperone would either be fully released from the nascent transporter, captured by the N domain of TAP1, or it may even be picked up by the N domain of TAP2. How could deficient tapasin binding impair the TAP1-TAP2 dimerization process? One plausible way would be by causing a lower rate of TAP dimer initiation (i.e., causing a lower rate of capturing TAP2 into the complex). Once fully synthesized, unassembled TAP2 loses its ability to form a dimer with TAP1 and is unstable and rapidly degraded (45); however, it remains possible that proper tapasin association is required to organize molecules within a rearranging TAP complex, and lack of this association may lead to subtly altered transporter dimers that are less stable.

The capacity of tapasin to stabilize TAP is well established (7–9, 26, 27, 28, 42), so it was not surprising to find that the decay rate of the TAP1 subunit depends on tapasin binding (Fig. 9A–E). However, earlier reports noted that unassembled rat-derived 1ΔN was surprisingly stable, raising speculations that it might be the binding of tapasin to the coreTMD that is key to stabilize TAP1 (18). We therefore compared in a purely human experimental system the stability of 1ΔN and TAP1-3AQ, lacking tapasin binding to one or the other binding site. Surprisingly, both constructs, when unassembled, were fairly stable, showing only a slightly faster decay than the wt subunit (Fig. 9C–E). This suggests that tapasin binding to either of the two docking sites is sufficient to significantly stabilize the protein. Strikingly, though, when binding to both sites was abolished as in constructs 1ΔN-3AQ and 1ΔN-5AQ, a drastic loss of stability was observed resulting in half-lives of only ~4 h (Fig. 9A, 9B). These results suggest that both tapasin binding sites in TAP1 may be functionally redundant to some extent with respect to stabilizing TAP, but that both sites cooperate to achieve the full stability of the molecule. In summary, our results demonstrate that human unassembled TAP1 interacts with tapasin via the coreTMD. We map this interaction to TM segment TM9 and find that within the respective amphipathic helix, it is the polar face that drives the association. Within this polar face, we identify critical amino acid residues necessary for tapasin binding. Experiments using mutant TAP1 subunits that are deficient in tapasin binding to the coreTMD suggest that this interaction plays a role in both heterodimerization and stabilization of the transporter. Thus, our results provide novel insights into the network of interactions that guide the assembly and biogenesis of TAP and the PLC, a process critical for MHC class I-mediated immunity.

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Disclosures
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