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*J Immunol* published online 31 August 2015
http://www.jimmunol.org/content/early/2015/08/30/jimmunol.1500985
Use of Functional Polymorphisms To Elucidate the Peptide Binding Site of TAP Complexes

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TAP1/TAP2 complexes translocate peptides from the cytosol to the endoplasmic reticulum lumen to enable immune surveillance by CD8+ T cells. Peptide transport is preceded by peptide binding to a cytosol-accessible surface of TAP1/TAP2 complexes, but the location of the TAP peptide-binding pocket remains unknown. Guided by the known contributions of polymorphic TAP variants to peptide selection, we combined homology modeling of TAP with experimental measurements to identify several TAP residues that interact with peptides. Models for peptide–TAP complexes were generated, which indicate bent conformation for peptides. The peptide binding site of TAP is located at the hydrophobic boundary of the cytosolic membrane leaflet, with striking parallels to the glutathione binding site of NaAtm1, a transporter that functions in bacterial heavy metal detoxification. These studies illustrate the conservation of the ligand recognition modes of bacterial and mammalian transporters involved in peptide-guided cellular surveillance.

The online version of this article contains supplemental material.

MHC class I molecules play a central role in the adaptive immune response to viruses and cancers by presenting antigenic peptides to CD8+ CTL (1, 2). TAP is localized on the ER membrane and required for the transport of cytosolic peptides into ER lumen for assembly with newly synthesized MHC class I molecules (3). Recent studies have suggested that TAP can also be localized on phagosomal membranes under certain conditions, to transport cytosolic Ags into phagosomes (4). Because of its essential role, TAP deficiency results in human immunodeficiency (5), and inhibition of TAP expression and function are common in viral infections and cancers (6).

TAP is a member of the superfamily of ABC transporters (ABC) that translocate a wide range of solutes across membranes using ATP hydrolysis as an energy source (7). TAP is composed of two homologous half-transporters, TAP1 (ABCB2) and TAP2 (ABCB3). TAP shares a common architecture with other ABC transporters, with two transmembrane domains (TMDs) formed by long TM α-helices that are extended into the cytosol and two cytosolic nucleotide-binding domains (NBDs) (Fig. 1). The TMD of each subunit has a core domain (core TMD) and an N-terminal accessory domain (TMD0) (8, 9). Two six-helical core TMDs of the TAP1/TAP2 complex contain the peptide-binding pocket and translocation channel and, together with NBDs, are essential and sufficient for peptide transport (8, 9).

Structural requirements and binding specificities for peptide substrates have been studied for TAP proteins from a number of different species (10–14). In general, the first three N-terminal and the last C-terminal residues of translocated peptides are most critical for peptide-binding specificity (12, 13, 15). Free N- and C-termini of peptide are also required for efficient TAP binding (10, 13, 16). The residues in the center of the peptide have small or no effects on the substrate specificity and transport efficiency of TAP (12, 13).

Despite some progress in identification of possible determinants of TAP peptide binding specificity (14, 17–21), the exact location of the peptide binding pocket within the TAP transport core domains and the preferred configurations of TAP-bound peptides are yet to be identified. In this study, we present homology models of TAP translocation complexes (excluding TMD0 domains) of rat, human, and two chicken TAPs of the B4 or B15 haplotypes (rat [r]TAP1/TAP2a, human [h]TAP1/TAP2, chicken [c]TAP1/TAP2-B4, and cTAP1/TAP2-B15) bound to their peptide substrates. Site-directed mutagenesis and chemical cross-linking were used to assess the proximity of peptides to TAP residues lining the internal cavity and to test the involvement of selected TAP residues upon peptide binding and transport. We used the current results together with previously published data to predict peptide docking modes within the ligand binding site of TAP transporters.

Materials and Methods

Molecular modeling

TAP sequences. Models of rat, human, and chicken TAP1/TAP2 heterodimers were built using sequences of rTAP1 (residues 147–725) and rTAP2a (residues 135–703) (UniProtKB access codes P36370 and P36372, respectively); hTAP1 (residues 170–748) and hTAP2 (residues 135–686) (UniProtKB access codes Q03518 and Q03519, respectively), and chicken TAP (residues 4–575 for cTAP1 and 133–701 for cTAP2) from alleles B4 and B15 (GenBank protein IDs AEE25613.1 and AEE25616.1 for TAP1 and AEE25620.1 and AEE25623.1 for TAP2).

Homology models were generated for the 12-helical core domain of TAP1/TAP2 heterodimers that represents the core peptide translocation unit shared with other ABC transporters. The N-terminal accessory domain (TMD0), which is not required for peptide translocation but which participates in binding MHC class I molecules via the assembly factor tapasin (8, 9), was not modeled because it lacks a structural template from...
a homologous protein domain. The sequence alignments of the core TMDs of TAPs modeled in this work are shown in Supplemental Fig. 1. Sequence alignments of NBD domains have been published previously (22).

Structural templates. Models of rat TAP core domains were constructed in both the inward-facing and the outward-facing conformations and carefully analyzed and refined in the context of experimental data. Other models were built only in the inward-facing conformation. The structural templates used for modeling represent crystal structures of bacterial ABC exporters with relatively low homology to TAP (25–28% residue identity) that were crystallized in different conformational states: structure of putative drug exporter Sav1886 (PDB ID: 2hyd) in outward-facing conformation (23) and structure of the heavy metal exporter NaAtm1 in inward-facing conformation (PDB ID: 4mrs) (24). Among numerous available crystal structures in inward-facing conformations (24–28), we chose the structural template of NaAtm1 transporter because it was cocrystallized with hexapeptide ligands, and the NBDs did not display wide separation. Recent electron single-particle cryomicroscopy study of a bacterial TmAB exporter in β-DDM micelles supported the functional relevance of C-terminally connected NBDs in the nucleotide-free inward-facing state (29).

Homology modeling. Homology models of rTAP1 and rTAP2a based on Sav1886 or NaAtm1 template were generated by Phyre2-server (30) using the sequence alignments mentioned above. Backbone corrections were introduced in some loops and helices to provide consistency between models generated from different templates. The structure of each half-transporter was refined by energy minimization (100 steps) with the CHARMM force field implemented in QUANTA (Accelrys) using a di-electric constant (ε) of 10 and the adopted-basis Newton–Raphson method. Models of other half-transporters were generated from NaAtm1-based models of rTAP1 and rTAP2a using residue substitutions and placement of PDB ID: 4hyd (from human M2D2) residues. Three-dimensional structures of different TAP1/TAP2 heterodimers, models of TAP1 and TAP2 were superposed with subunits A and B, respectively, from structures of bacterial exporter dimers. Structures of different TAP heterodimers were further refined by loop correction and side-chain adjustment, mainly at dimerization interfaces.

Model assessment. Developed models of TAP transport domains closely reproduced structures of their templates. In particular, root-mean-square deviations (RMSD) between models of TAP1 and TAP2a and 4mrs structures were 1.08 Å (for 684 Cα-atoms) and 0.29 Å (for 752 Cα-atoms), respectively. Model assessment by MolProbity (31) indicated the satisfactory quality of the TAP models. For example, Ramachandran maps of 2hyd- and 4mrs-based models of rTAP1 and rTAP2a co in complex with the FITC-labeled TR peptide showed that 90.7% (1038 of 1145) and 95.7% (1102 of 1151), respectively, of all residues were in favored regions, 98.3% of residues were in allowed regions with templates with in template maps. The MolProbity score of 4mrs-based model was 1.94 (79th percentile among 27675 X-ray structures) based on deviations in main chain geometry (<0.3Å) and Cβ geometry (<0.6Å) and the clash score of 4.24 (96th percentile). To additionally verify correctness of the models of core TMDs, we analyzed locations of evolutionarily conserved residues in transporter sequence and structure (Supplemental Fig. 1A). Residues that are conserved in TAP sequences are located exclusively at the interhelical interfaces of each half-transporter in either inward-facing or outward-facing conformation (residues shown by sticks on panels B and C). These residues likely participate in structure stabilization of each half-transporter. Eleven residues are highly conserved in sequences of not only TAPs but also other ABC transporters (indicated by asterisk in Supplemental Fig. 1A). Five of these residues from TM1/TM6 contact within each subunit at both membrane sides, which may be essential for structural stabilization of moving elements of these helices. Six highly conserved residues are located between TAP1 and TAP2: between TM2 and CL-2 and between CL-2 and NBD (by sticks-and-spheres and marked by circles/ovals on Supplemental Fig. 1B, 1C). These residues likely play an essential role in stimulation of the ATPase activity of NBDs by peptides bound to TMDs.

Peptide docking. Structures of peptide ligands were based on conformations of fragments from templates in different conformations that were taken from the crystal structure of the membrane β-barrel, VDAC1 (PDB ID: 3emm) (32). Specifically, to model the bent conformation of 8- and 9-mer, we used β-hairpin fragment 172–180 of the 3emm structure, and for modeling 10-mer, we used β-hairpin 224–233, which has a β-bulge. The peptide modeling included residue substitutions followed by energy minimization with QUANTA/CHARMM (100 steps, using 10 of the adopted-basis Newton–Raphson method). Initial docking of low-energy conformations of the TR nonapeptide in the bent conformation into rTAP1/TAP2a complex was performed manually to satisfy four major structural constraints from this study: 1 and 2)
heads (Qiagen), beads were washed with lysis buffer, and proteins were eluted by SDS sample buffer. Ten microliters of each sample was loaded and resolved by SDS/PAGE and visualized on a Typhoon scanner (at 520 nm). The relative fluorescence intensity B was quantified from the fluorimages and plotted as a function of peptide concentration [P]. Data were fitted using Equation: B = Bmax[P]/(Kd+[P]), and Kp values were determined by nonlinear least squares analysis. It is possible that the use of the cross-linker in these assays interferes with the derivation of true Kp values reflective of steady state binding. We thus use the term apparent Kd (Kd,app) throughout the study.

Results

Homology modeling of the core transport unit of TAP complexes and mapping of polymorphic residues containing determinants of peptide selectivity

TAP from different species and their allelic variants differ in their peptide transport specificities. rTAP2 has two allelic variants, a and u, whereas hTAP and murine TAP (mTAP) have no functional polymorphism. rTAP1/TAP2a complexes are rather promiscuous and allow the transport of peptides with basic C-terminal amino acid ("Arg-permissive") as also seen with hTAP (hTAP1/TAP2) (12, 13, 16). In contrast, rTAP1/TAP2u complexes and mTAP1/TAP2 complexes are restrictive for the transport of peptides with charged residues, but permissive to bulky aliphatic and aromatic residues ("Arg-restrictive") (10, 16). In addition, cTAP1 and cTAP2 sequences exhibit high sequence polymorphisms and distinct preferences for transport of certain peptide sequence motifs (14). These polymorphic residues are expected to be important in peptide recognition.

In the absence of crystal structures of full-length TAP complexes with and without substrate, homology models of TAP in different functional states are useful for predicting peptide binding modes and uncovering molecular determinants of the selective peptide binding and transport. We thus constructed homology models of the TAP core transport units of rTAP1/TAP2a (Fig. 1A–C) and cTAP1/TAP2-B4 (Fig. 1D) (validations of model quality are described in Materials and Methods). All models were built using the crystal structure of the homodimeric bacterial exporter NaAtm1 which represents the inward-facing conformation with two separated NBD domains that contact only through their C-terminal helices (24) (Fig. 1A, 1C, 1D). In addition, rTAP1/TAP2u was modeled in the outward-facing conformation (Fig. 1B), using the structural template of the ADP-bound bacterial drug exporter Sav1866 (23). In the latter structure, the NBDs dimerize forming one consensus and one degenerate ATPase sites at their dimerization interfaces (33), which in the models are occupied by ADP molecules (Fig. 1B).

Both TAP conformations showed spacious internal cavities near the cytosolic membrane leaflet that are lined mostly by polar and charged residues, which form extensive hydrogen-bonding networks and could also interact with polar groups of bound peptides (Fig. 1A, 1B, red ovals). Attempts to identify determinants of TAP selectivity have suggested that polymorphic rat TAP2 residues at positions 217/218, 262, 265 and 266, and 374/380 might determine the differential transport of peptides with C-terminal arginine (18, 20). In homology models of rTAP1/TAP2a complexes, residues 218, 262, 265, and 266 of rTAP2a face the internal cavity of TAP1/TAP2 heterodimer (Fig. 1C). Several cTAP polymorphic residues (131, 179, 289, of cTAP1 and 216, 263, 378, and 416 of cTAP2 that correspond to positions 278, 326, and 436 of rTAP1 and 218, 265, 380, and 418 of rTAP2) also face the internal cavity (Fig. 1D). Almost half of all polymorphic rat and chicken residues are located near the cytosolic membrane boundary (Fig. 1C, 1D), where the size and the shape of the internal cavity are rather similar in both inward-facing and outward-facing conformations of the transporter (Fig. 1A, 1B, red ovals). We hypothesized that this region may represent the binding pocket for peptides, which preferentially interact with the inward-facing conformation of TAP in a nucleotide-independent manner.

Glu218 of rTAP2a controls specific translocation of Arg-terminated peptides

To elucidate the functional role of polymorphic rTAP residues located near the internal cavity, we generated single mutants of rTAP2a at positions 218, 262, 265, and 266, in which the residues were mutated to the corresponding residues of rTAP2u and co-expressed the mutants with rTAP1. A pair of peptides labeled by FITC that differ only at their C termini, TVDNK*TAYR (TR) and TVDNK*TAYV (TV), were used in peptide transport assays.

As shown in the peptide transport analysis (Fig. 2A, 2B), rTAP1/TAP2a(Q262R), rTAP1/TAP2a(S265P), and rTAP1/TAP2a (L266F) displayed transport efficiencies quite similar to the wild-type rTAP1/TAP2a complex for both peptides. In contrast, the E218M mutation in TAP2a that eliminates the local negative charge in the TM2 strongly decreased the transport efficiency of the TR peptide, which has a positively charged arginine at the C terminus (Fig. 2A), but did not significantly affect the translocation of the TV peptide, which has a hydrophobic C-terminal valine (Fig. 2B). The selectivity differences of this single mutant resemble the selectivity differences between rTAP1/TAP2a and rTAP1/TAP2u complexes. These results indicated that E218 of rTAP2a is a key residue involved in determining the substrate transport specificity at the peptide C terminus. To address whether other polymorphic rTAP2 residues have synergistic effects, we introduced a combination of four mutations within TM2 and TM3, which resulted in the quadruple mutant construct rTAP1/TAP2a (E218M/Q262R/S265P/L266F) in the peptide transport assay, the quadruple mutant showed a similar functional fingerprint as the single rTAP1/TAP2a(E218M) mutant (Fig. 2A, 2B). In addition, a stronger effect of the quadruple mutant on reducing transport efficiency of the TR peptide compared with the single rTAP1/TAP2a(E218M) mutant was noted, indicating some contribution of TM3 residues in recognition of peptide C terminus. Similar results were obtained for the effects of the single (E218M) and quadruple (E218M/Q262R/S265P/L266F) mutations in rTAP2a upon transport of a pair of decapeptides, RYWANATK*SR (RR) and RYWANATK*SF (RF). The effects of the mutations were significant for the RR peptide but not the RF peptide (Fig. 2C, 2D). These results confirmed the critical role of the single E218 residue in selective translocation of multiple peptides bearing C-terminal basic residues. All mutants were expressed at similar levels as their wild-type rTAP counterparts (Fig. 2E), suggesting that the mutations do not compromise TAP folding, as also indicated by full competence for the transport of the TV and RF peptides by the mutants.

Cys273 of rTAP1 cross-links to residue 6 of a nonpeptide

ATP-independent peptide binding to TAP precedes the ATP-dependent peptide translocation step. Peptide transport efficiency usually correlates with peptide-binding affinity (35). However, impaired transport might also relate to the obstruction of TMD conformational changes (36, 37). Therefore, a direct peptide binding study is essential to determine whether E218 of rTAP2a is a component of the peptide-binding pocket. We thus developed a fluorescent peptide-based cross-linking assay to examine peptide binding.

The transport unit of the rTAP contains 14 cysteine residues, 10 of which are located within the core TMDs (Fig. 3A). Two of these cysteines line the internal cavity in the modeled inward-
facing TAP conformation: C273 of rTAP1 and C362 of rTAP2a. We predicted that sulphydryl groups of cysteine-containing peptides could be conjugated with free thiol groups of one of these two cysteine residues via a chemical cross-linker. For assessing cross-linking to cysteine-containing peptides, we used two FITC-labeled TR peptides with cysteine substitutions at either the N-terminal P2 position (peptide TC2R) or central P6 position (peptide TC6R) and the homobifunctional cross-linker BMOE with spacer arm length of 8 Å, which can irreversibly cross-link two cysteine residues. After cross-linking, rTAP complexes were recovered from microsome lysates by Ni-NTA beads and loaded on a SDS-PAGE gel. Cross-linked TAP was visualized by fluorimaging of gels. We found that only the TC6R peptide was cross-linked to rTAP1/TAP2a (Fig. 3B). Notably, an excess of the competitor TR peptide blocked the cross-linking, confirming the specificity of the reaction (Fig. 3C). On the basis of the TAP specificity of the reaction (Fig. 3B, 3C), in subsequent experiments, we did not purify TAP from microsomes, and instead directly loaded the total microsome lysates on gels for fluorimaging.

To further investigate which cysteine residue within the internal cavity of the wild-type rTAP becomes conjugated with TC6R, two mutant constructs (rTAP1[C273A] and rTAP2a[C362A]) were generated and tested for cross-linking to the TC6R peptide. In addition, as a negative control, we generated the rTAP1(C175A) mutant with alanine-substituted cysteine in a region outside of the predicted peptide-binding pocket (Fig. 3A). We found that only the rTAP1(C273A) mutant completely abolished the ability of the rat TAP complex to be linked to TC6R via BMOE in the absence of ATP, even under conditions of slightly higher expression compared with wild-type TAP complexes (Fig. 3D). In contrast, the rTAP1(C175A)/TAP2a and the rTAP1/TAP2a(C362A) mutants retained the ability to crosslink with the TC6R peptide (Fig. 3D). The peptide transport efficiency of rTAP1(C273A)/TAP2a was similar to that of wild-type TAP complex (Fig. 3E), indicating that impaired cross-linking to this mutant complex is not related to protein misfolding. These results demonstrated that the central region (P6) of the nonameric peptide is located close to C273 of rTAP1.

Glu218 of rTAP2a determines the specific binding of Arg-terminated peptides

We used the cross-linking assay described above to further evaluate the peptide binding by different TAP constructs (in the absence of
ATP). By quantifying the fluorescence intensities, the averaged $K_{D,app}$ value of the TC6R peptide for rTAP1/TAP2a was calculated to be $186.6 \pm 23.2$ nM. Cross-linking of the TC6R peptide was largely impaired for the E218M and the quadruple mutants of rTAP2a (Fig. 4A), indicating decreased binding efficiencies of the TC6R peptide to both mutants ($K_{D,app}$ could not be accurately determined). These results are in line with the significant impairment in TR transport in both cases (Fig. 2). In contrast, the binding efficiencies of the TC6V peptide to rTAP1/TAP2a(E218M) ($K_{D,app} = 339.2 \pm 50.6$ nM) or the quadruple mutant complexes ($K_{D,app} = 107.3 \pm 5.8$ nM) were not reduced compared with wild-type rTAP ($K_{D,app} = 416.7 \pm 115.0$ nM) (Fig. 4B). These experiments provide direct evidence that E218 of rTAP2a is a key residue that determines ATP-independent binding of peptides with basic C-terminal residues.

Tyr385 of rTAP1 is a general determinant of peptide binding that favors interactions with peptides containing hydrophobic C-termini

TAP transporters across many species are permissive for the transport of peptides with C-terminal basic residues (16). We found that TAP1-Y385 in TM5, which is spatially proximal to TAP2a-E218 from TM2 (Fig. 5A), is conserved across TAP1 from many species. To investigate whether Y385 of rTAP1 determines TAP preference for peptides with hydrophobic C termini, we examined peptide transport by the rTAP1(Y385A)/TAP2a mutant complex. As shown in Fig. 5B, there was a significant reduction in the transport efficiency of the TV peptide, and a less significant reduction in transport of the TR peptide by rTAP1(Y385A)/TAP2a compared with the wild-type TAP. We also found that the binding to rTAP1(Y385A)/TAP2a of TC6V peptide was decreased relative to those observed with the rTAP1/TAP2a complexes, again to a greater extent compared with that observed with the TC6R peptide (Fig. 5C, 5D; $K_{D,app}$ values for rTAP1[Y385A]/TAP2a could not be accurately assessed).

To assess peptide translocation by the rTAP1(Y385A)/TAP2a and the rTAP1/TAP2a(E218M) mutant complexes for a wider range of peptides, we examined peptide transport inhibition, using permissive or partially permissive peptides as reporters: TV for rTAP1/TAP2a(E218M) and TR for rTAP1(Y385A)/TAP2a (Fig. 5E, 5F), and two libraries of nonapeptides (X₈R and X₈F), which had random amino acids (Cys excluded) at positions 1–8 and a fixed basic (Arg) or hydrophobic (Phe) C-terminal residue. We found that transport of TV by the rTAP1(Y385A)/TAP2a complexes was more efficiently inhibited by peptide libraries with a C-terminal Phe than with C-terminal Arg (IC₅₀s were 1.4 and 28.6 µM, respectively) (Fig. 5E). Transport of TR by rTAP1(Y385A)/TAP2a complexes was better inhibited by a peptide library with C-terminal Arg than Phe (IC₅₀ were 1.5 and >10 µM, respectively) (Fig. 5F).
However, both X8Ra and X8F peptide libraries inhibited binding of TR and TV peptides to rTAP1/TAP2a to more similar extents (IC50 between 1.4 and 4.7 μM).

These results imply that both E218 of TAP2a and Y385 of TAP1 are functionally important in determining the transport specificities of a range of peptides. TAP2a-E218 specifically favors transport of peptides with basic C-terminal residues, whereas Y385 of TAP1 favors transport of peptides with hydrophobic C termini. We also suggest that TAP1-Y385 is more important for TAP function than TAP2a-E218 because mutations of TAP1-Y385 resulted in reduced binding and transport of peptides with both basic and hydrophobic C-terminal residues.

Proximity of rTAP1-E436 to the peptide N terminus supports a model for a bent conformation of TAP-bound peptides

The experimental results thus far provided three anchor points for a peptide substrate inside the rTAP internal cavity: direct contacts of peptide C-terminal side chains with rTAP2a-E218 and rTAP1-Y385 and covalent linking of peptide P6 with rTAP1-C273. On the basis of this information, we docked the FITC- and BMOE-labeled TC6R peptide into the TAP complex. The presence of two bulky covalently bound probes, FITC linked to K5 and BMOE linked to C6 of the peptide, significantly increases the peptide volume and restricts its orientation inside the internal cavity. These spatial restrictions and three identified anchoring points allowed us to unambiguously define the position of the peptide C terminus but not
of the N terminus. The N-terminal part of a peptide could adopt quite different conformations and orientations within the large peptide translocation channel, either pointing toward cytosol in an extended, quasi-linear conformation or being directed toward the luminal side in a bent configuration (Fig. 6A).

To localize the N terminus of the TAP-bound peptide inside the ligand-binding pocket and examine the preferred (bent versus linear) conformation of the TAP-bound peptides, we performed additional cross-linking assays with the TC2R peptide containing a cysteine at the peptide residue 2 (C2). We designed rTAP mutants by introducing cysteines into positions 385 or 436 of rTAP1, which are predicted to be close to the C2 of TC2R in a bent conformation (Cp-Cp distances in the inward-facing state rTAP model are \( \sim 2-3 \) Å, respectively). We also designed mutants with cysteines at positions 263 of rTAP1 and 251 of rTAP2a, predicted to be close to C2 of TC2R peptide in a linear conformation (Cp-Cp distances are \( \sim 4-5 \) Å, respectively). We expected that cross-linking of TC2R with either C436 or C385 of rTAP1 would support a bent conformation for the peptide, whereas cross-linking with either C263 of rTAP1 or C251 of rTAP2a would point to a linear peptide conformation. To avoid cross-linking to other cysteine residues in the vicinity of the cavity, the cysteine mutants were generated on the rTAP1(C273A)/TAP2a(C362A) background (named rTAP1\(^{-}\)/TAP2a\(^{-}\)) to remove the other cysteines that face the internal cavity of the TAP heterodimer (Fig. 3A).

We found that the TC2 peptide could be conjugated via BMOE only to rTAP1\(^{-}\)(E436C)/TAP2a\(^{-}\) but not to rTAP1\(^{-}\)(S263C)/ TAP2a\(^{-}\) or rTAP1\(^{-}\)/TAP2a\(^{-}\) (S251C) (Fig. 6B). This result provided direct evidence supporting a bent model of the TAP-bound peptide. There was no significant cross-linking between TC2R and TAP1-C385 of rTAP1\(^{-}\)(Y385C)/TAP2a\(^{-}\), which is also predicted to be in cross-linking proximity to peptide residue 2 in a bent conformation. The significant impairment in peptide binding that results from rTAP1\(^{-}\)(Y385) mutations (Fig. 5) could explain this result. In contrast, the lack of cross-linking of rTAP1\(^{-}\)/TAP2a\(^{-}\) (S251C) or rTAP1\(^{-}\)(S263C)/TAP2a\(^{-}\) mutants to TC2R could not be explained by the impaired binding of these mutants because transport and binding of the TR peptide by rTAP1/TAP2(S251A) and rTAP1(S263A)/TAP2 complexes were not reduced relative to the wild-type TAP (Supplemental Fig. 2). Cross-linking of rTAP1\(^{-}\)(E436C)/TAP2a\(^{-}\) was selective toward peptide TC2R but not TC6R (Fig. 6C) verifying the closer proximity of rTAP1-436 to the peptide N terminus compared with the central residues of the peptide.

The rTAP1\(^{-}\)(E436C)/TAP2a\(^{-}\) complex, which was efficiently cross-linked to TC2R, demonstrated partial reduction in transport of both TR and TV peptides as compared with the rTAP1\(^{-}\)/ TAP2a\(^{-}\) complex (Fig. 6D). rTAP1-E436 is a site of polymorphism in cTAP (R289; Fig. 1D). To further explore the role of acidic TAP1-E436 residue in plausible interactions with peptides, we designed the rTAP1(E436R) mutant with a charge inversion and assessed binding and transport of four FITC-labeled peptides that carried different net charges (TV: \(-1\), TR: 0, RF: +1, RR: +2). We found a 2-fold increased translocation efficiency of the negatively charged TV peptide and reduced transport of neutral TR peptide (by \( \sim 28\% \)) (Fig. 7A). The rTAP1(E436R)/TAP2a complexes also showed reductions in transport efficiencies for the basic RR and RF peptides compared with the wild-type TAP complex (by \( \sim 43 \) and \( \sim 45\% \), respectively) (Fig. 7B). In the peptide binding assessments, although the differences between wild-type TAP and rTAP1(E436R)/TAP2a appeared to be small (Fig. 7C, 7D), in pairwise comparisons within experiments, both TC6R and TC6V displayed higher binding affinities to rTAP1 (E436R)/TAP2a complexes compared with wild-type complexes (\( \sim 28\% \) reduction in TV binding efficiency). In parallel experiments, we found that the RR and RF peptides inhibited cross-linking of peptide TC6R to rTAP1(E436R)/TAP2a with higher IC\(_{50}\) values compared with those for the wild-type TAP complex (Fig. 7E, 7F), indicating reduced binding affinities of both RR and RF peptides to rTAP1(E436R)/TAP2a.

Thus, the wild-type TAP complex with negatively charged rTAP1-E436 preferentially binds the positively charged peptides (RR and RF). A TAP mutant with a basic R436 at the same position favors the binding and transport of the negatively charged TV peptide. These results indicate that the TAP-E436 residue is a determinant for recognition of peptide charge, further discussed below based on modeling.
Modeling of peptide–TAP complexes

The 9-mer peptide could be unambiguously modeled inside the ligand pocket in a bent configuration based on identification of key anchor points for the TAP-bound nonapeptide, including 1) cross-linking between TC2R and the rTAP1(E436C) mutant (Fig. 6B), 2) cross-linking between TC6R and rTAP1-C273 (Fig. 3D), and 3) demonstration of contributions of rTAP2a-E218 and rTAP1 (Y385A)/TAP2a complexes. To model bent peptides inside the translocation channel, we chose \( \beta \)-hairpin–like peptide conformations stabilized by internal hydrogen bonds as an

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**FIGURE 5.** Key role of rTAP1-Y385 in the transport and specific recognition of peptides with hydrophobic C termini. (A) Location of rTAP1-Y385 (T1Y385) in the inward-facing rat TAP model. rTAP1-C273 (T1C273) and rTAP2a-E218 (T2E218) are indicated as reference points. Residues are shown as sticks. (B) Transport of peptides TR (TVDNK*TAYR) and TV (TVDNK*TAYV) (K* represents lysine-FITC) by wild-type rTAP1/TAP2a and rTAP1 (Y385A)/TAP2a complexes. Data are averages of three independent experiments, each in triplicate, with two different microsome preparations. Statistical analyses are based on paired two-tailed \( t \) tests. *** \( p \leq 0.001 \). Error bars represent SEM values. (C and D) Impaired binding of peptides TVDNK*CAYR (TC6R) (C) and TVDNK*CAYV (TC6V) (D) to rTAP1(Y385A)/TAP2a complexes, using BMOE-based cross-linking as described in Figs. 3 and 4. Insets show representative immunoblots. Data are from five or two independent analyses for TC6R and TC6V, respectively. (E and F) Effects of rTAP2a(E218M) (E) and rTAP1(Y385A) (F) mutations on peptide transport inhibition by the indicated peptide libraries X8R and X8F, where X indicates 19 randomized amino acids excluding cysteine. Data are representative of four (rTAP1/TAP2a[E218M]) or two (rTAP1[Y385A]/TAP2a) independent experiments each performed in duplicate with a single microsome preparation.
FIGURE 6. Proximity of rTAP1-E436 to the peptide N terminus supports a model for a bent peptide conformation. (A) Models for linear (left) and bent (right) conformations of the TVDNK*TAYR (TR) peptide in the translocation channel of the [TAP1/TAP2a] model in an inward-facing conformation. TM helices are shown as cartoons. Residues that could anchor the C terminus, P2, or P6 of the peptide are shown by sticks. The TR peptide is also shown by sticks. Interactions with side chains of V2 and R9/R10 and rTAP1-Y385. In addition, in the RR model, H-bonding and ionic contacts are formed between peptide R1 and rTAP1-E436 and with rTAP2a-E166 and rTAP2a-E418. Thus, the RR decapeptide has more contacts within the modeled ligand-binding pockets than the TR nonapeptide, which could explain the higher binding affinity of RR compared with TR observed in inhibition experiments (Fig. 7E; IC50 of 0.3 μM for RR but >2 μM for TR [data not shown]). On the basis of the models of mutant rTAP1(E436R)/TAP2a complex, RR and RF peptides likely encounter ionic repulsions due to close proximity between R1 and TAP1-R436 (Fig. 8B), which could explain the reduced binding and transport activities of RR and RF peptides by rTAP1 (E436R)/TAP2a relative to wild-type TAP (Fig. 7B, 7E, 7F). In contrast, TR and TV might form favorable ionic interactions between their D3 and rTAP1-R436 in rTAP1(E436R)/TAP2a mutant (Fig. 8A), which could explain the tendency toward improved binding affinities of nonapeptides to the mutant TAP, as discussed above (Fig. 7C, 7D). During transition of TAP to the outward-facing conformation, all electrostatic and hydrophobic interactions of TR and TV seem to be weakened, based on the models (Supplemental Fig. 3). Thus, TV, with fewer ionic interactions near the C terminus compared with TR (Fig. 8A), may dissociate more readily than TR, thereby increasing transport efficiency in the context of rTAP1(E436R)/TAP2a complexes (Fig. 7A).

To identify additional determinants of TAP translocation specificity, we constructed models of peptide–transporter complexes for two cTAPs from haplotypes B4 and B15, which have been shown to be selective for acidic (ADYNDSAE) and basic (KRYNASAY) peptides, respectively (14). As expected, substitutions in polymorphic residues of TAP1 and TAP2 sequences correlate with distinct sequence preferences for transported peptides. In particular, the exclusive acidic residues at positions 2, 5, and 8 of ADYNDSAE form specific ionic interactions with basic residues energetically preferred structure in an environment with the lowered dielectric constant (ε~10) that is expected inside the translocation channel. Validations of model qualities are described in Materials and Methods.

The TR peptide docked into the inward-facing rTAP1/TAP2a complex in a β-hairpin–like conformation is surrounded by 13 TAP residues (Fig. 8A). The involvement of seven of these residues (C273, Y385, and E436 of rTAP1, and E218, Q262, S265, and L266 of rTAP2a) in the proposed peptide binding pocket is supported by our current experiments. Four residues (D278 and Y326 of rTAP1 and E418 and Q424 of rTAP2a) are variable in rat or chicken TAP1 and TAP2 sequences, which suggests their role in selective recognition of different peptide ligands. Three residues, E381, Y385 of rTAP1 and Y428 of rTAP2a, are conserved across species and may be essential for the peptide translocation along the channel. Interactions of peptide with these three conserved residues are different in models of TR–TAP complexes in the inward-facing and the outward-facing states (Supplemental Fig. 3). For example, rTAP1-Y385 forms hydrophobic interactions with side chains of V2 and R9 in the inward-facing state model but becomes more distant from V2 in the outward-facing state model. Similarly, only in the inward-facing state model, π-stacking interactions are formed between aromatic rings of rTAP2a-Y428 and peptide Y8, and rTAP1-E381 forms H-bonds with peptide R9. Alterations in the interactions of peptide with these residues of TAP in the outward-facing state compared with the inward-facing state could reduce the binding affinity, and initiate the release of peptide into the ER lumen.

In the models of rTAP1/TAP2a with docked FITC-labeled TR and RR peptides (Fig. 8A, 8B), electrostatic (H-bonding and ionic) interactions are formed between peptide R9/R10 and TAP2a-E218 and between the peptide N terminus (NH2 +) and rTAP1-E436, whereas hydrophobic interactions are formed between peptide R9/R10 and rTAP1-Y385. In addition, in the RR model, H-bonding and ionic contacts are formed between peptide R1 and rTAP1-E436 and with rTAP2a-E166 and rTAP2a-E418. Thus, the RR decapeptide has more contacts within the modeled ligand-binding pockets than the TR nonapeptide, which could explain the higher binding affinity of RR compared with TR observed in inhibition experiments (Fig. 7E; IC50 of 0.3 μM for RR but >2 μM for TR [data not shown]). On the basis of the models of mutant rTAP1(E436R)/TAP2a complex, RR and RF peptides likely encounter ionic repulsions due to close proximity between R1 and TAP1-R436 (Fig. 8B), which could explain the reduced binding and transport activities of RR and RF peptides by rTAP1 (E436R)/TAP2a relative to wild-type TAP (Fig. 7B, 7E, 7F). In contrast, TR and TV might form favorable ionic interactions between their D3 and rTAP1-R436 in rTAP1(E436R)/TAP2a mutant (Fig. 8A), which could explain the tendency toward improved binding affinities of nonapeptides to the mutant TAP, as discussed above (Fig. 7C, 7D). During transition of TAP to the outward-facing conformation, all electrostatic and hydrophobic interactions of TR and TV seem to be weakened, based on the models (Supplemental Fig. 3). Thus, TV, with fewer ionic interactions near the C terminus compared with TR (Fig. 8A), may dissociate more readily than TR, thereby increasing transport efficiency in the context of rTAP1(E436R)/TAP2a complexes (Fig. 7A).
FIGURE 7. Effects of the rTAP1(E436R) mutation on transport (A and B) and binding (C–F) of peptides with different net charges (q). (A) The rTAP1(E436R) mutation reduces transport efficiency for the neutral peptide TVDNK*TAHY (q = 0) but increases transport efficiency for the negatively charged peptide TVDNK*TAYR (q = -1). (B) The rTAP1(E436R) mutation reduces the efficiency of transport of both basic peptides RYWA-NATK*SF (q = +1) and RYWANATK*SR (q = +2). Peptide transport data are averages of three independent experiments each in triplicate with two (A) or a single microsome preparation (B). (C–F) The rTAP1(E436R) mutation increases binding affinities of the negatively charged TVDNK*CAYV (TC6V) (D) nonapeptides but decreases the binding of two basic decapeptides, RYWANATKSR (RR) (E) and RYWANATKSF (RF) (F). Peptide binding of nonapeptides (C and D) was assessed by direct BMOE-mediated cross-linking assays as described in Materials and Methods. Derived apparent peptide binding affinities from independent experiments for rTAP1(E436R)/TAP2a and wild-type TAP complexes were respectively: TC6R, 14.8 ± 9.8 nM versus 106.9 ± 25.6 nM, 36.6 ± 13.4 nM versus 109.7 ± 25.5 nM, 18.9 ± 11.1 nM versus 104.4 ± 11.1 nM, 23.7 ± 8.1 nM versus 43.9 ± 10.9 nM, 90.2 ± 27.9 nM versus 93.9 ± 13.7; p = 0.0345. TC6V, 263.2 ± 55.7 nM versus 410.1 ± 143.1 nM, 397.6 ± 50.6 nM versus 790.4 ± 121.9 nM, 345.5 ± 65.1 nM versus 894.0 ± 505.2 nM, 318.0 ± 105.1 nM versus 649.3 ± 248.6 nM; p = 0.0067). Peptide binding of decapeptides (E and F) was measured by their competitive inhibition of binding of TC6R (1 μM). K* represents lysine-FITC. For (C–F), representative data of two independent experiments with a single microsome preparation are shown. Error bars represent SEM values. Statistical analyses for (A–D) are based on paired two-tailed t tests. *p ≤ 0.05, **p ≤ 0.01.

of TAP from B4 haplotype, cTAP1-R289 (rTAP1-E436), cTAP1-K131 (rTAP1-D278), and cTAP2-R216 (rTAP2a-E218), respectively (Fig. 8C). In contrast, the essential basic and aromatic residues at positions 1, 2, and 8 of the double-labeled nonapeptide was around 22 Å (38). We analyzed orientations and distances between nitroxyl radicals of proxyl-labeled side chains at positions 2 and 8 of the double-labeled nonapeptide was around 22 Å (38). We analyzed orientations and distances between nitroxyl radicals of proxyl-labeled side chains at positions 2 and 8 of the double-labeled nonapeptide was around 22 Å (38). We analyzed orientations and distances between nitroxyl radicals of proxyl-labeled side chains at positions 2 and 8 of the double-labeled nonapeptide was around 22 Å (38).
with the electron paramagnetic resonance data, although the bent conformation in our model contradicts the author’s conclusions about plausible extended conformation of the TAP-bound peptide (38).

**Discussion**

An understanding of the peptide translocation specificity of TAP is largely impeded by the absence of experimental structures of the full-length transporters in complexes with peptide substrates. On the basis of the results described in this study, we constructed three-dimensional models of peptide–TAP complexes for rat, chicken, and human TAPs (Fig. 8, Supplemental Figs. 3, 4), which were extensively validated in the context of known experimental data and functional polymorphism of rat and chicken TAPs. Our model of rat TAP in the outward-facing state is similar to previously published Sav1866-based models of the human TAP (22, 37). In contrast, the inward-facing conformation of our TAP complexes significantly differ from previously published human TAP models that were produced based on P-glycoprotein, MsbA, and ABCB10 crystal structures (22).

Our models of peptide–TAP complexes for different species are mainly based on four interaction sites for TAP-bound peptides elucidated from this work (residues C273, Y385, and E436 of rTAP1 and E218 of rTAP2a) (Fig. 8, Supplemental Figs. 3, 4). In all the models, TAP-bound peptides are located at ~7 Å distances from both sides of the cytosolic membrane boundary (Fig. 9A). In the NaAtm1 structural template, two molecules of cocrystallized GSSG occupy a similar position (Fig. 9B). The location of TAP-bound peptide also overlaps with the binding site of an unknown molecule that was copurified with the TmrAB dimer (29) but differs from positions of P-glycoprotein ligands that are inserted deeper into membrane-embedded TMD domains, 5–10 Å away from the cytoplasmic surface (39).

We found strong inhibition of binding and translocation of peptides with C-terminal arginines by rTAP complexes with either a single TAP2a(E218M) or the quadruple mutant (Figs. 2, 4). It is known that the presence of Met at TAP2-218 position coincides with “Arg-restrictive” phenotypes of mTAP1/TAP2 and rTAP1/T234 or rTAP1-E381, and cTAP1-R/E289 or rTAP1-E436) and TM2 and TM6 of TAP (cTAP2-R/G216 or rTAP2-E/M218, cTAP2-K/N416 or rTAP2-E418, and cTAP2-Q422 or rTAP2-Q424) are likely to function as determinants of selective peptide recognition (Fig. 8, Supplemental Figs. 3, 4). In particular, we found that a charged residue from the TM6 of TAP1 (rTAP1-E436 or cTAP1-R/E289)
participates in charge recognition at the peptide N terminus, and therefore plays an important role for TAP translocation selectivity.

Notably, the localization of bound RRYQKSTEL in our model of the ATPase sites in an asymmetric ABC transporter MsbA: Alternating access with a twist. J. Biol. Chem. 24: 51–62.


