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Use of Functional Polymorphisms To Elucidate the Peptide Binding Site of TAP Complexes

Jie Geng,*†,1 Irina D. Pogozheva,†,1 Henry I. Mosberg,† and Malini Raghavan*

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 Journal of Immunology, 2015, 195: 000–000.

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 ATP binding cassette (ABC) transporters 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 (ABC2B) and TAP2 (ABC2B3). 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

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Abbreviations used in this article: ABC, ATP binding cassette; BMOE, bismaleimide-dithiane; cTAP, chicken TAP; ER, endoplasmic reticulum; hTAP1/TAP2, human TAP; hTAP2, human TAP2; mTAP, murine TAP; NBD, nucleotide-binding domain; rTAP1/TAP2, rat TAP; TMD, transmembrane domain.

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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 transporters 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 NusAtm1 in inward-facing conformation (PDB ID: 4msr) (24). Among numerous available crystal structures in inward-facing conformations (24–28), we chose the structural template of NusAtm1 transporter because it was co-crystallized with hexapeptide ligands, and the NBDs did not display wide separation. Recent electron single-particle cryo-microscopy study of a bacterial TmrAB 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 NusAtm1 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 model and crystal structures of 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 dielectric constant (ε) of 10 and the adopted-basis Newton–Raphson method. Models of other half-transporters were generated from NusAtm1-based models of rTAP1 and rTAP2a using residue substitutions and placement of the core NBD sequence in the corresponding software house (Supplemental Fig. 1). Three-dimensional structures of different TAPI/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 between model and template structures were 1.08 Å (for 684 Ca-atoms) and 0.29 Å (for 752 Ca-atoms), respectively. Model assessment by MolProbity (31) indicated the satisfactory quality of the TAP models. For example, Ramachandran maps of 2hyd- and 4msr-based models of rTAP1 and rTAP2a using residue substitutions and placement of the core NBD sequence in the corresponding software house (Supplemental Fig. 1) were in close agreement to the corresponding software house. The MolProbity score of 4msr-based model of TAP was 1.94 (79th percentile) and the adopted-basis Newton–Raphson method). After minimization of the TAP1-TAP2 complex the main-chain geometry of 9- and 10-mer peptides did not significantly differ from the geometry of parent β-hairpins from the VDAC1 structure: r.m.s.d. between Ca-atoms of residues 2–9 of 9-mer (TVDNKTYAR, RRYQKSTEL, and their derivatives) and residues 173–180 of the 3Emn structure were <0.6 Å, whereas r.m.s.d. between Ca-atoms of residues 2–10 of the 10-meric RWYANATKSR and residues 275–283 of the 3Emn structure were <0.6 Å.

The coordinate files (in PDB format) of the 2hyd- and 4msr-based models of rTAP1/TAP2a in complex with FITC-labeled TVDNKTYAR, peptide can be downloaded from the Web site: http://mosberglab.phar.unich.edu/resources/. Other developed models can be obtained upon request.

**Baculovirus constructs**
PFastBac Dual vector encoding both wild-type rTAP1 and rTAP2a was gifted by Dr. R. Gaudet (Harvard University, Cambridge, MA) (33). Mutations were made using QuickChange II Site-Directed Mutagenesis Kits (Agilent Technologies). All the primers were purchased from Invitrogen. All sequences were confirmed by DNA sequencing at the University of Michigan Sequencing Core. Baculovirus stocks were prepared according to the Bac-to-Bac manual (Invitrogen).

**Insect cell infections, microsome preparations, and peptide translocation**
S21 cells were cultured in Grace’s insect medium (Invitrogen), supplemented with 10% FBS. The cells were grown to confluence and infected with the appropriate baculoviruses at a multiplicity of infections of 5–20, depending on the protein expression level of individual baculoviruses. Following these infections, the cells were harvested after 72 h and microsomal membranes were generated as described previously (34). Protein expression was analyzed by immunoblotting assays with anti-His or anti-Flag Abs for rTAP1 and rTAP2, respectively. Peptide transport assays were performed by incubating microsomes with 1 μM FITC-labeled peptide (from Peptide 2.0) at 37°C for 5 min in the presence of absence of 5 mM ATP. Microsomes were lysed in 1% Nonidet P-40 and the fraction of transported and glycosylated peptide was recovered with Con A–Sepharose (Pharmacia, Freiburg, Germany). Fluorescence signals obtained in the presence and absence of ATP were measured in triplicate using a plate reader (Bio-Tek, Synergy 2), and the +ATP/−ATP ratios were obtained. To compute transport efficiencies, +ATP/−ATP ratios from multiple independent experiments were normalized, setting values from microsomes expressing wild-type rTAP at 100% and control microsomes lacking TAP at 0%, and averaged. Data are plotted as the mean ± SEM of measurements of indicated replicates. Statistical analyses are based on paired two-tailed t tests using Prism 6 (GraphPad, La Jolla, CA). For competition assays, peptide libraries XXXXXXXX (X=R) and XXXXXXXX (X=K) (where X stands for randomized 19 aa excluding cysteine) were synthesized by Sigma-Aldrich. Unlabeled competitor peptide in the appropriate concentration, or buffer for the controls, was added to fluorescent peptide and microsome suspensions to a final volume of 100 μL in the presence or absence of ATP. The concentration needed for 50% inhibition of fluorescent peptide transport was determined using Prism 6 (GraphPad, La Jolla, CA).

**Cross-linking assays**
Microsomes were incubated with cysteine-containing fluorescently labeled peptides on ice for 10 min in PBS (pH 7.4), in a 100-μL reaction volume. BMEO (Pierce) was added to a final concentration of 0.2 mM. After a 10 min incubation at room temperature, cross-linking was quenched by adding 10 mM DTT. Products were further purified by Ni-NTA beads or directly used for SDS-PAGE. For purification on nickel beads, microsomes were lysed in 1% Nonidet P-40 and TAP complexes were bound to Ni-NTA
heads (Qiagen), beads were washed with lysis buffer, and proteins were eluted by SDS sample buffer. Ten microfils 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 fluorimaging and plotted as a function of peptide concentration [P]. Data were fitted using Equation: $B = B_{max}[P]/(K_D + [P])$, and $K_D$ 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 $K_D$ values reflective of steady state binding. We thus use the term apparent $K_D$ 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 alphatic 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) (validation of models quality are described in Materials and Methods). All models were built using the crystal structure of the homodimeric bacterial exporter NapA (23). The transport unit of the rTAP contains 14 cysteine residues, 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 TAP2 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 TAP2a 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...
facing TAP conformation: C273 of rTAP1 and C362 of rTAP2a. We predicted that sulhydryl 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, of the two FITC-labeled peptides, only TC6R 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.

Glu$^{218}$ 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

FIGURE 1. Localization of polymorphic residues in models of rat and chicken TAP1/TAP2 complexes. Homology models of the core transport domains of rat TAP complexes in the inward-facing (A, C, and D) or the outward-facing (B) conformations were built using crystal structures of bacterial ABC transporters, NaAtm1 (PDB ID: 4mrs) and Sav1866 (PDB ID: 2hyd), respectively. Models are colored blue (for TAP1) and yellow (for TAP2). Membrane boundaries for template structures are taken from the OPM database (http://opm.phar.umich.edu) and shown by red and blue dotted lines. Cartoon representations show the architecture of TAP composed of two TMDs and two NBDs. TM helices of rTAP2a are indicated by numbers. ADP molecules bound to NBDs in the outward-facing conformation (B) are shown by spheres colored green. All polymorphic residues from TMDs of rat TAP (rTAP1/TAP2a) (C) and chicken TAP from haplotype B4 (cTAP1/TAP2-B4) (D) are shown by spheres colored blue for residues from TAP1, and orange for residues from TAP2. Red ovals show plausible peptide binding sites.
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 \(\mu\)M, respectively) (Fig. 5E). Transport of TR by rTAP1(Y385A)/TAP2a complexes was better inhibited by a peptide library with C-terminal Arg than Phe (IC50s were 1.5 and >10 \(\mu\)M, 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...
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 (C∼b-Cb distances in the inward-facing state rTAP model are ∼4 and ∼9 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 ∼4 and ∼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 TC2R 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 or 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 rTAP (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 ∼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 ∼43 and ∼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 (KD,app values derived from individual experiments are listed in the legends to Fig. 7C, 7D). In parallel experiments, we found that the RR and RF peptides inhibit cross-linking of peptide TC6R to rTAP1(E436R)/TAP2a with higher IC50s 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 binding of peptide C terminus (Figs. 4, 5). To model bent peptides inside the translocation channel, we chose \( \beta \)-hairpin–like peptide conformations stabilized by internal hydrogen bonds as an

**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 \( X_{R}R \) and \( X_{F}F \), 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.

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-Y385 to binding of peptide C terminus (Figs. 4, 5). To model bent peptides inside the translocation channel, we chose \( \beta \)-hairpin–like peptide conformations stabilized by internal hydrogen bonds as an
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. Cross-linking between C2 of TCDNK*TAYR (TC2R) peptide (at 1 μM; K* represents lysine-FITC) and indicated TAP complexes (top panel). Data are representative of four experiments with two different microsome preparations. Middle and bottom panels show representative immunoblots. (B) Cross-linking occurred between rT1*(E436C)/T2* and C2 of TC2R peptide but not C6 of TVDNK*CAYR (TC6R) (at 1 μM; K* represents lysine-FITC) (top panel). Data are representative of four experiments with a single microsome preparation. Middle and bottom panels show representative immunoblots. (C) Impaired transport of TR and TV peptides by the rT1*(E436C)/T2* mutant. Data are averages of three experiments each in triplicate, with two different microsome preparations. All constructs shown in the figure are on the rTAP1(C273A)/TAP2a(C362A) background (labeled as T1*/T2*). Error bars represent SEM values. Statistical analyses are based on paired two-tailed t tests. **p ≤ 0.01, ***p ≤ 0.001.

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 (NH₃⁺) 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). 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...
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*TAYR (q = 0) but increases transport efficiency for the negatively charged peptide TVDNK*TAYV (q = -1). (B) The rTAP1(E436R) mutation reduces the efficiency of transport of both basic peptides RYWANATK*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 ± 6.6; 106.9 ± 65.1 nM versus 27.9 nM versus 43.9 ± 10.9 nM, 90.0 ± 27.9 nM versus 93.9 ± 13.7; p = 0.0345. TC6V, 263 ± 55.7 nM versus 410 ± 143.1 nM, 397.4 ± 50.6 nM versus 790.4 ± 121.9 nM, 345 ± 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 KRYNASAY interact with residues of TAP from B15 haplotype, cTAP1-D234 (rTAP1-E381), cTAP1-E289 (rTAP1-E436), and cTAP2-G216 (rTAP2a-E218) of B15 haplotype, respectively (Fig. 8D). These results indicate that several residues from TM3, TM5, and TM6 of TAP1 (positions 278, 381, and 436 in rTAP1) could represent important determinants of selective recognition of peptide motifs. It has been previously reported that the nonapeptide RRYQKCTEL with a cysteine at the P6 position cross-links with the chemical protease (36). Recent electron paramagnetic resonance studies showed that interspin distance between spin probes 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 TAP-bound spin-labeled RCYQKSTCL peptide by docking the peptide into the TAP model, based on the docking mode discussed in Fig. 8A. We found that the conformation and placement of the spin-labeled nonapeptide and its long proxyl-modified side chains are restricted inside the peptide binding pocket. Therefore, the extended configuration of proxyl spin probes, their opposite orientations, and rather large distance between nitroxyl groups (22.6 Å) are unequivocally defined (Supplemental Fig. 4C). The obtained distance is in agreement

resulting models, BM[PEO]$_3$ is in sufficient proximity to V288C to form a covalent linkage with this cysteine in TM3 of hTAP1 (Supplemental Fig. 4A), in agreement with the previous experimental data (36). In addition, BABE is positioned near residue 290 of hTAP1 (Supplemental Fig. 4B), consistent with the previous experimental identification of the site of polypeptide cleavage by the chemical protease (36).
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).

Further analyses of peptide docking in models of chicken and rat TAPs indicate that several residues from the TM3, TM5, and TM6 of TAP1 (cTAP1-K/E131 or rTAP1-D278, cTAP1-D234 or rTAP1-E381, and cTAP1-R/E289 or rTAP1-E436) and TM2 and TM6 of TAP2 (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 RYRQKSTEL in our model of human TAP-peptide complex (Supplemental Fig. 4) significantly differs from the peptide positioning proposed by Corradi et al. (22), using homology modeling and replica exchange simulations in the absence of experimental data. The Corradi model, based on the structure of the ABCB10 in the inward-facing conformation, places the peptide in an extended conformation in the large cytoplasmic opening between both TAP subunits, ~10–15 Å below the cytosolic membrane border. The latter model, which was not based on experimental constraints, is inconsistent with several components of the experimental data described in this paper, and does not explain the peptide repositioning in the outward-facing state of the transporter, when a large part of the proposed peptide binding site becomes occluded by moving TM helices.

Based on the current modeling studies, we propose that the peptide, in a β-hairpin–like conformation, binds to the ligand binding site of the nucleotide-free inward-facing conformation of TAP, which is located between TM helices at the cytoplasmic membrane boundary (Fig. 9). Peptide binding to TMDs facilitates the transition of the transporter to the outward-facing state (40). In that state, the peptide likely maintains a bent conformation as well as its overall position within the translocation channel but may have altered interactions with TAP residues (Supplemental Fig. 3), which would facilitate peptide release from the TMD translocation channel to the ER lumen.

In conclusion, this study provides structural models for the TAP–peptide complexes in different functional states of the transporters that were extensively validated using current and previous experimental data. These models can be used to guide future discovery of TAP inhibitors for treatment of autoimmune diseases.

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Disclosures

The authors have no financial conflicts of interest.

References