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

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

*J Immunol* published online 31 August 2015
http://www.jimmunol.org/content/early/2015/08/30/jimmunol.1500985

Supplementary Material  
http://www.jimmunol.org/content/suppl/2015/08/30/jimmunol.1500985.DCSupplemental

Subscription  
Information about subscribing to *The Journal of Immunology* is online at:  
http://jimmunol.org/subscription

Permissions  
Submit copyright permission requests at:  
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts  
Receive free email-alerts when new articles cite this article. Sign up at:  
http://jimmunol.org/alerts

*The Journal of Immunology* is published twice each month by  
The American Association of Immunologists, Inc.,  
1451 Rockville Pike, Suite 650, Rockville, MD 20852  
Copyright © 2015 by The American Association of Immunologists, Inc. All rights reserved.  
Print ISSN: 0022-1767 Online ISSN: 1550-6606.
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 cancers and viruses 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 (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 rTAP1/TAP2a, human hTAP1/TAP2, chicken cTAP1/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 cocystalized with hexapeptide ligands, and the NBDs did not display wide separation. Recent electron single-particle cryomicroscopy 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 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 and templates 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 di-electric constant (ε) of 10 and the adopted-basis Newton–Raphson method. Models of other half-transporters were generated from NaAtm1-template models of rTAP1 and rTAP2a using residue substitutions and placement of NBDs. Using our custom-made software, we identified 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 of rTAP1/TAP2 and 4mrs structures were 1.08 Å (for 684 Cα-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 4mrs-based models of rTAP1/TAP2a 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% (Supplemental Fig. 1A). Five highly conserved residues are served 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 were conserved in all different conformational states of the core TMDs. Conformations of rTAP1/TAP2a and 4mrs-based models were 0.6 Å, whereas r.m.s.d. between Cα-invariant residues of 2- and 9-mer (TVDNKTAYR, RRYQKSTEL, and their derivatives) and residues 173–180 of the tetratricopeptide structure were <0.6 Å, whereas r.m.s.d. between Ca-atoms of residues 2–9 of the 9-mer (TVDNKTAYR, RRYQKSTEL, and their derivatives) and residues 173–180 of the 4mrs structure were <0.6 Å, whereas r.m.s.d. between Ca-atoms of residues 2–10 of the 10-mer RYYWANATKSR and residues 275–283 of the 4mrs structure were ~0.9 Å.

The coordinate files (in PDB format) of the 2hyd- and 4mrs-based models of rTAP1/TAP2a in complex with FITC-labeled TVDNKTAYR peptide can be downloaded from the Web site: http://mosberglab.phar.umich.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. Follicular cells from 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 microbodies with 1 μM FITC-labeled peptide (from Peptide 2.0) at 37°C for 5 min in the presence or 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 rTAP1 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 Prizm 6 (GraphPad, La Jolla, CA, software). For competition assays, peptide libraries XXXXXXXXSR (X=xR) and XXXXXXXXXSR (X=xF) (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 Prizm 6 (GraphPad, La Jolla, CA, software).

**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. BMOE (Pierce) was added to a final concentration of 0.2 mM. After 10 min reaction 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
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, we generated single mutants of 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. 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. 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, we generated single mutants of 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 and 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 complexes (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/TAP2a 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 (13, 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.

Glu<sup>218</sup> 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.

Cys<sup>273</sup> 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. All polymorphic residues from TMDs of rat TAP (rTAP1/rTAP2a) (C) and chicken TAP from haplotype B4 (cTAP1/rTAP2-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.

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/TAP2 was calculated to be 186.6 ± 23.2 nM. Cross-linking of the TC6R peptide was largely impaired for the E218M and the quadruple mutants of rTAP2 (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 ± 50.6 nM) or the quadruple mutant complexes ($K_{D,app}$ = 107.3 ± 5.8 nM) were not reduced compared with wild-type rTAP ($K_{D,app}$ = 416.7 ± 115.0 nM) (Fig. 4B). These experiments provide direct evidence that E218 of rTAP2 is a key residue that determines ATP-independent binding of peptides with basic C-terminal residues.

Tyr$^{385}$ 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 (IC$_{50}$s 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 (IC$_{50}$ 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.

**FIGURE 3.** Proximity of P6 of a TAP-bound nonapeptide to rTAP1-C273. (A) Locations of cysteine residues (shown by spheres) of rTAP1 (colored dark gray) and rTAP2a (colored light gray) subunits of the Na2ATM1-based homology model. Three cysteine residues mutated in this work are indicated in larger size. Positions of membrane boundaries are as defined in Fig. 1. (B) Top panel, Prominent BMOE-mediated cross-linking of wild-type (shown as WT) rTAP1/TAP2a to a fluorescent peptide containing a cysteine at position 6 [TVDNK*KAYR (TC6R)] compared with that observed with peptide bearing a cysteine at position 2 [TCDNK*TAYR (TC2R)] at peptide concentration of 1 μM (K* represents lysine-FITC). Cross-linked products were loaded directly on SDS-PAGE gels (left lanes) or further purified using Ni-NTA beads before loading (right lanes). Data are representative of two independent experiments, each in duplicate. TAP expression was confirmed by immunoblotting (middle and bottom panels). (C) Peptide TC6R (1 μM) cross-linking with wild-type TAP was blocked by peptide TVDNKTAYR. Representative data of two independent experiments with two microsome preparations are shown. (D) Mutation at TAP1-C273 disrupted TC6R peptide cross-linking. Top panel, Cross-linking between TC2R or TC6R (1 μM) and wild-type or mutants, rTAP1(C175A)/TAP2a (T1C175A/T2), rTAP1/TAP2a(C362A) (T1/T2C362A), and rTAP1(C273A)/TAP2a (T1C273A/T2). Data are representative of four independent experiments. Middle panel, T1C273A/T2 does not cross-link with TC6R even at higher peptide concentrations. Data are representative of two independent experiments. Bottom panel, Protein expression levels by immunoblots. (E) Similar expression levels and transport activities of mutants and wild-type rTAP1/TAP2a were confirmed by immunoblotting (top panel) and peptide transport assays (bottom panel, average of two independent transport assays with two different microsome preparations). Error bars represent SEM values. Differences were not statistically significant, based on paired two-tailed t tests.

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 TAP 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 (Cβ-Cβ distances in the inward-facing state rTAP model are ∼7 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 (Cα-Cα 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 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 ∼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 (K_{D,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 IC_{50S} 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 β-hairpin–like peptide conformations stabilized by internal hydrogen bonds as an...
The Journal of Immunology 9

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*KAYR (TR) peptide in the translocation channel of the (TAPI/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. (B) Cross-linking between C2 of TCDNK*KAYR (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. (C) Cross-linking occurred between rT1*(E436C)/T2* and C2 of TC2R peptide but not C6 of TVDNK*KAYR (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. (D) 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.

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 rTAPI/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 rTAPI, 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 rTAPI 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 rTAPI and Y428 of rTAP2a, are conserved across species and may be essential for the peptide translocation along the channel. Interactions of peptide with these conserved residues are different in models of TR–TAP complexes in the inward-facing and the outward-facing states (Supplemental Fig. 3). For example, rTAPI-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 rTAPI-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 rTAPI/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 R1 and TAP1-R436, and between the peptide N terminus (NH2+) and rTAPI-E436, whereas hydrophobic interactions are formed between peptide R9/R10 and rTAPI-Y385. In addition, in the RR model, H-bonding and ionic contacts are formed between peptide R1 and rTAPI-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 rTAPI(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 rTAPI (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 rTAPI-R436 in rTAPI(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 rTAPI(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 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 rTAPI(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 rTAPI (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 rTAPI-R436 in rTAPI(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 rTAPI(E436R)/TAP2a complexes (Fig. 7A).
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 interspin distance between spin probes at positions 2 and 8 of the double-labeled nonapeptide and its long proxyl spin-labeled nonapeptide 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
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 cytoplasmatic 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/TAP2u complexes (20). Comparison of models of chicken TAPs from haplotypes B4 and B15 (Fig. 8C, 8D) also emphasized a role for residue 216 from cTAP2 (position 218 in rTAP2) in the specific recognition of peptide C-terminal residues. In particular, it became clear from the models that basic R216 forms an ionic pair with acidic E8 residue of octapeptides, whereas G216 favorably interacts with peptide with a bulky aromatic Phe residue at peptide C terminus (Fig. 8C, 8D).

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 RRYQKSTEL 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.

Acknowledgments
This work used the DNA sequencing and Hybridoma Cores of the University of Michigan. We thank Dr. Rachelle Gaudet for the pFastBac Dual vector encoding wild-type rTAP1 and rTAP2a and Dr. Andrew Tai (University of Michigan Medical School, Ann Arbor, MI) for the use of the Synergy 2 plate reader.

Disclosures
The authors have no financial conflicts of interest.

References


Use of functional polymorphisms to elucidate the peptide binding site of TAP complexes

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

Supplemental data
Figure S1. Evolutionarily conserved residues in TAP TMDs. (A) Alignments of sequences of TAP core TMDs from rat (rTAP), human (hTAP), chicken (cTAP) TAPs with structural templates used for transporter modeling (4mrs and 2hyd PDB entries). Locations of helices in structural templates are marked by gray. Polymorphic residues in sequences of rat TAPs and chicken TAPs are marked by green. Residues from the proposed ligand binding pocket are marked by black; residues mutated in this work are marked by red. Conserved residues of TAPs are colored blue (for TAP1) and orange (for TAP2). Residues conserved in diverse ABC-transporters are indicated by asterisks and marked by red circles, for residues participating in intersubunit interactions (B, C) Location of conserved residues in 4mrs-based (B) and 2hyd-based (C) models of rTAP1/TAP2a. Helices of TAP TMDs are shown by ribbons; conserved residues are shown by sticks colored blue (for rTAP1) and orange (for rTAP2a) and marked by spheres for residues conserved in ABC-transporters. FITC-labeled TR ligand is shown by spheres colored cyan, residues mutated in this work are shown by red sticks with labels. Highly conserved residues that form intersubunit interactions are marked by red ovals. Positions of hydrophobic membrane boundaries were calculated by PPM server.
Figure S2. Effects of rTAP1(S263A) and rTAP2a(S251A) mutations on peptide transport (A) and binding (B). Transport efficiency for the TVDNK*TAYR peptide and binding ability of the TVDNK*CAYR peptide (K* represents lysine-FITC) were assessed as described in Methods. Peptide transport data are average of three independent experiments each in triplicate with 2 different microsome preparations and peptide binding data are representative of 2 independent experiments with a single microsome preparation.
Figure S3. Ligand binding pocket of rTAP1/2a (cross-eyed stereo pair). 4mrs-based (A, B) and 2hyd-based (C, D) models are shown from the ER lumenal membrane side (A, C) and cytoplasmic side (B, D). Helices are shown by cartoons colored blue (rTAP1) and yellow (rTAP2a), FITC-labeled TVDNK*TAYR ligand is shown by sticks with C-atoms colored purple, residues from the binding pockets are shown by sticks with C-atoms colored blue (for TAP1) and yellow (for TAP2a); O-atoms are colored red, N-atoms are colored blue. Hydrogen bonds formed between main-chain atoms of ligand and between ligand and transporter side chains are shown by gray dashes. Residue contacts that are significantly changed during conformational transitions are marked by yellow ovals.
**Figure S4.** Models for hTAP1/TAP2 in inward-facing conformations with bound nonapeptides. (A) Model of the hTAP1(V288C)/TAP2 with RRYQKCTEL modified by the 14.7 Å-long crosslinker BM[PEO], that connects C6 of the peptide to TAP1(V288C). (B) Model of the hTAP1/TAP2 with RRYQKCTEL modified at C6 by the iron-dependent protease BABE, which cleaves TAP1 polypeptide chain near TAP1-E290. (C) Conformation of the spin-labeled nonapeptide RCYQKSTCL in the binding pocket of the hTAP1/TAP2 complex in the inward-facing conformation. Docking of the peptide modified by bulky proxyl labels restrict the conformation and orientation of proxyl labels, which leads to an interspin distance at 22.6 Å, similar to the experimentally determined value. TM helices of the hTAP core are presented by a cartoon colored blue (for TAP1) and yellow (for TAP2); peptide is shown by sticks with C-atoms colored cyan, BM[PEO], BABE, and proxyl labels are shown by sticks with C-atoms colored purple. Residues in the ligand binding pocket are shown by sticks with C-atoms colored blue (for TAP1) and yellow (for TAP2). Residues at positions 213, 217, and 374 of TAP2 are located outside the peptide binding pocket.