Catalytic Site Modifications of TAP1 and TAP2 and Their Functional Consequences*

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The transporter associated with antigen processing (TAP), a member of the ATP binding cassette (ABC) family of transmembrane transporters, transports peptides across the endoplasmic reticulum membrane for assembly of major histocompatibility complex class I molecules. Two subunits, TAP1 and TAP2, are required for peptide transport, and ATP hydrolysis by TAP1-TAP2 complexes is important for transport activity. Two nucleotide binding sites are present in TAP1-TAP2 complexes. Compared with other ABC transporters, the first nucleotide binding site contains non-consensus catalytic site residues, including Asp668 in the Walker B region of TAP1 (in place of a highly conserved glutamic acid), and Gln701 in the switch region of TAP1 (in place of a highly conserved histidine). At the second nucleotide binding site, a glutamic acid (TAP2 Glu632) follows the Walker B motif, and the switch region contains a histidine (TAP2 His661). We found that alterations at Glu632 and His661 of TAP2 significantly reduced peptide translocation and/or TAP-induced major histocompatibility complex class I surface expression. Alterations of TAP1 Asp668 alone or in combination with TAP1 Gln701 had only small effects on TAP activity. Thus, the naturally occurring Asp668 and Gln701 alterations of TAP1 are likely to contribute to attenuated catalytic activity at the first nucleotide binding site (the TAP1 site) of TAP complexes. Due to its enhanced catalytic activity, the second nucleotide binding site (the TAP2 site) appears to be the main site driving peptide transport. A mechanistic model involving one main active site is likely to apply to other ABC transporters that have an asymmetric distribution of catalytic site residues within the two nucleotide binding sites.

Cytotoxic T lymphocytes identify and eliminate cells harboring pathogens by monitoring peptide-major histocompatibility complex (MHC)³ class I molecules at the cell surface. The transporter associated with antigen processing (TAP) is critical for antigen presentation by MHC class I molecules (1). TAP

translocates antigenic peptides from the cytosol into the lumen of the endoplasmic reticulum (ER), where peptides are loaded onto MHC class I molecules. Deletion of TAP or certain mutations of TAP severely affect translocation of peptides into the ER, resulting in marked reduction in MHC class I surface expression due to defects in their assembly (2).

TAP belongs to the large family of ABC transporters, a number of which are associated with severe human diseases like cystic fibrosis (3). ABC transporters have a common architecture of two transmembrane domains, which are responsible for substrate translocation, and two nucleotide-binding domains (NBDs), which hydrolyze ATP to provide energy required for translocation of substrates. TAP consists of two subunits, TAP1 (ABCB2) and TAP2 (ABCB3), each containing a transmembrane domain and an NBD. TAP1 and TAP2 form a complex that is necessary and sufficient for peptide translocation across the ER membrane. The NBDs of both TAP1 and TAP2 contain sequence motifs that are common to all ABC transporters. These include the Walker A and Walker B motifs, which are involved in ATP binding and hydrolysis, and the signature (Consensus C) motif. The Walker A motif has the consensus sequence GX₄GK(S/T) (X = any amino acid), and the Walker B motif has the consensus sequence ϕₓD (ϕ = hydrophobic amino acid). The signature motif is located between the Walker A and Walker B motifs, and has the consensus sequence LSGGQ. Other conserved motifs within ABC transporter NBD sequences include the switch region that is C-terminal to the Walker B region, and the Q loop, which is downstream of the Walker A region. In the transition state, nucleotides are thought to be sandwiched within an NBD dimer, between the Walker A residues of one NBD and signature residues of the second NBD (4–9). Two nucleotide binding sites are present within an NBD dimer. In the case of TAP complexes, the first nucleotide binding site (the TAP1 site) is expected to include residues from the Walker A regions of TAP1 and the signature region of TAP2 (Fig. 1A). The second site (the TAP2 site) is expected to include residues from the Walker A region of TAP2 and the signature region of TAP1 (Fig. 1B). Other conserved residues that are predicted to be in position to interact with the γ-phosphate (either directly or through a water molecule) include a glutamic acid residue immediately C-terminal to the Walker B motif, a highly conserved histidine in the switch region, and a glutamine residue within the Q loop (Fig. 1) (6, 10).

The glutamic acid residue C-terminal to the Walker B motif, and the highly conserved histidine in the switch region have been suggested to play key roles in ATP hydrolysis by ABC transporters (6, 11–16). In a majority of TAP1 sequences, the
conserved glutamic acid is changed to an aspartic acid (Asp<sup>668</sup> in human TAP1), and the conserved histidine is changed to a glutamine (Glu<sup>701</sup> in human TAP1) (Fig. 1). To further understand the functional consequences of these naturally occurring sequence alterations in TAP1, and the role of the TAP1 and TAP2 nucleotide binding sites in the TAP catalytic cycle, we generated and characterized the functions of TAP mutants with altered Asp<sup>668</sup> and Gln<sup>701</sup> of TAP1, and the counterpart generated and characterized the functions of TAP mutants.

**EXPERIMENTAL PROCEDURES**

**Baculovirus Constructs**—Baculoviruses encoding a histidine-tagged version of human TAP1 (TAP1-his), a green fluorescent protein-tagged version of human TAP2 (TAP2-eYFP) were obtained as described (17, 18). Baculoviruses encoding untagged human TAP1 and TAP2 were obtained from Dr. Robert Tampe (19), and a virus encoding a yellow fluorescent protein-tagged version of human TAP1 (TAP1-eGFP), and a green fluorescent protein-tagged version of human TAP2 (TAP2-eYFP) were obtained from Dr. Rachelle Gaudet (20). Site-directed mutations (D668N in TAP1; E632Q in TAP2) were introduced into the TAP1-his and TAP2 constructs in the pPCR2.1 vector (17) using the QuikChange site-directed mutagenesis kit. The mutations were introduced using the Stratagene QuikChange site-directed mutagenesis kit. For TAP1, the primers were: 5′-GGGTCCTCATTCTAGATCAGGCTAC-3′ and 3′-CATCACCGCGCACCTCAGCCTG-3′ (reverse primer). For TAP2, the primers were: 5′-GGGTCTGACCTACTATGACGGTCATGAGGTCATGAGGTCAGTACTGCCCCTAG-3′ and 3′-CAGGCGACTGTTGCCATTATCTTAGATAAGTACAC-3′ (reverse primer). Silent mutations were introduced into both primers to allow for easy identification of mutant clones. All the primers were purchased from Invitrogen. Mutated cDNAs were sequenced, excised from pPCR 2.1 (using BamHI and BglII, respectively, for TAP1 and TAP2), and ligated into pAcUW51 transfer vector. The transfer vectors and BaculoGold DNA (BD Pharmingen) were co-transfected into insect cells as described in the Baculovirus Expression Manual (BD Pharmingen). Plaque assays were used to isolate pure clones, which were used to reinfect cells for virus amplifications.

**A**  
**TAP1 Walker A**  
**TAP2 Signature Motif**  

**B**  
**TAP2 Walker A**  
**TAP1 Signature Motif**  

FIGURE 1. Predicted architectures of the TAP1 (A) and TAP2 (B) nucleotide binding sites in the transition state, based upon structural studies with MJ0796 (6) and other ABC transporters. Residues predicted to be involved in nucleotide contacts from the Walker A and the Signature motif end are indicated for each site, and residues involved in protein-protein contacts are indicated in the extreme left of each panel. The GPNGSKST sequence is the Walker A motif. Gln<sup>586</sup> and Glu<sup>587</sup> of TAP1, and Gln<sup>551</sup> and Glu<sup>552</sup> of TAP2, form the Q loops. Asp<sup>668</sup> of TAP1 and Glu<sup>632</sup> of TAP2 are found immediately after the Walker B sequences. Gln<sup>701</sup> of TAP1 and His<sup>661</sup> of TAP2 are from the switch regions.

**Insect Cell Infections, Microsome Preparations, TAP Expression Analyses, Peptide Translocation, and Peptide Binding Assays**—SF21 cells were cultured in Grace’s insect medium (Invitrogen), supplemented with 10% fetal bovine serum. The cells were grown to confluence and infected with the appropriate baculovirus at a multiplicity of infection of 5–30, depending on the protein expression level of individual baculovirus. Following these infections, the cells were harvested after 72 h and microsomal membranes were generated as described (17, 19). TAP1 expression in the microsomes was verified by immunoblotting analyses with the 148.3 hybridoma supernatant (TAP1-specific antibody) (19), or purified anti-TAP1 (obtained from Dr. Soldano Ferrone), or (for TAP1-eGFP) 2 µl of anti-GFP ascites fluid (Covance Scientific). TAP2 expression was detected using 435.3 hybridoma supernatant (21). Peptide transport was analyzed using radiolabeled peptide RR<sup>125I</sup>NASTEL as described previously (17, 19). The peptide binding assays were carried out using established procedures with the fluorescent peptide RRYQKCTTC.TEL (17, 22).

**Photoaffinity Labeling with 8-Azido-ATP**—Labeling with 8-azido nucleotides and binding analyses were undertaken as previously described (18).

**Retroviral Constructs and Analyses of Retrovirus-infected Cells**—Desired TAP mutants were generated using the Stratagene QuikChange site-directed mutagenesis kit. The mutations for TAP1(D668N/Q701A) were done using TAP(D668N) in pACUW51 as the template in the mutagenesis reaction and the following two mutations primers: Q701A forward, 5′-CTTTCTCATCCACCGGACACCCCTAGCTGCTCCGGGTCAGTCTCAG-3′ and 5′-CTAGGCGACTGTTGCCATTATCTTAGATAAGTACAC-3′ (reverse primer). Silent mutations were introduced into both primers to allow for easy identification of mutant clones. All the primers were purchased from Invitrogen. Mutated cDNAs were sequenced, excised from pPCR 2.1 (using BamHI and BglII, respectively, for TAP1 and TAP2), and ligated into pAcUW51 transfer vector. The transfer vectors and BaculoGold DNA (BD Pharmingen) were co-transfected into insect cells as described in the Baculovirus Expression Manual (BD Pharmingen). Plaque assays were used to isolate pure clones, which were used to reinfect cells for virus amplifications.

**Experimental Procedures**

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CTG-3’-Q701H reverse, 5’-CAGGCTAGGGTGGTGGATGAGAAGGAG-3’. All TAP1 sequences were verified following the mutagenesis reactions. Sequences encoding TAP1-his, TAP1-(D668N), TAP1(D668N/Q701A), and TAP1(D668E/Q701H) were excised from pCR2.1 or pACUW51 using BamHI and inserted into the BglII site of pMSCVpuro (Clontech), a retroviral vector that contains a puromycin resistance gene.

The TAP2(E632Q/H661A) was generated using TAP2(E632Q) in pCR2.1 vector as the template. The primers used were H661A forward, 5’-CTGTTAGTTGCTCCAGGCCTGAGACA-3’ and H661A reverse, 5’-TGTCAGAGCTGCCAGAAAATCCAG-3’. TAP2(E632D) and TAP2(E632Q/H661Q) were expressed in insect cells along with the part- ers encoding TAP2 or its mutants.

Viruses were prepared in BOSC cells using previously established procedures (23). 100-mm plates were seeded with 2.5×10^6 cells and incubated at 37 °C for 24 h. In a sterile microcen- trifuge tube, 600 μl of Opti-MEM (Invitrogen) and 20 μl of FuGENE 6 (Roche) were incubated for 5 min at room tem- perature. 0.5 μg of vesicular stomatitis virus DNA and 5.5 μg of MSCV/TAP DNA were added to the Opti-MEM and FuGENE, and incubated for 20 min at room temperature. The media was removed from the BOSC cells down to 2 ml, and the DNA mixture was added dropwise to cover the whole plate. 6 ml of fresh Dulbecco’s modified Eagle’s medium (Invitrogen) containing 10% dialyzed fetal bovine serum (Invitrogen) was added and incubated at 37 °C for 30 min. Cells were trypsinized, centrifuged, and resuspended in 1 ml of methionine and cysteine-deficient Dulbecco’s modified Eagle’s medium. Cells were labeled with 0.2 μCi of [35S]methionine/cysteine (ICN) for 2 h. The cells were lysed with lysis buffer (1% Triton X-100, 10 mM Tris, 130 mM NaCl, complete EDTA-free protease inhibitors (Roche)) for 45 min before centrifuging for 20 min. Lysates were incubated for 45 min with 25 μl of 435.3 (21) supernatants or with anti-TAP2 antisera. 20 μl of protein G beads were added, and incubated at 4°C for an additional 45 min. The beads were washed three times with lysis buffer. SDS-PAGE was added, and samples were boiled for 5 min. The immunoprecipitated proteins were separated by 10% SDS-PAGE gels. Gels were dried for 1 h and exposed for 24 h. A Typhoon phosphorimager (Amersham Biosciences) was used to visualize immunoprecipitated proteins. A faint background band that migrated at a similar position as TAP2 was visualized in immunoprecipitates of the parent STF-1 cells. This background band is not TAP2 as the truncated endogenous TAP2 expressed in STF-1 cells has an expected molecular mass of 30 kDa (stop codon follows residue 273 of TAP2 (25)), which should not be recognized by the 435.3 antibody or anti-TAP2 antisera that are directed against TAP2 NBD.

RESULTS

The TAP2(E632Q) Mutation Impacts Peptide Translocation More Significantly Than the TAP1(D668N) Mutation—A histi- dine-tagged version of mutant TAP1(D668N) and untagged TAP2(E632Q) were expressed in insect cells along with the partner wild type subunits, using baculoviruses encoding the desired proteins. Microsomes were prepared of TAP1(D668N)/ TAP2, TAP1-TAP2(E632Q), or TAP1(D668N)-TAP2(E632Q) combinations, or wild type proteins, under conditions in which comparable levels of wild type or mutant proteins were expressed. Peptide transport by TAP complexes is preceded by peptide binding to TAP, a step that requires the membrane-spanning domains of both TAP1 and TAP2. Peptide binding to TAP is nucleotide binding independent at low temperatures (26, 27), and mutations targeting residues involved in γ-phosphate contact were not expected to affect peptide binding by TAP. Consistent with this expectation, a determination of KD values for binding of a fluorescent peptide substrate to TAP1(D668N)/TAP2 or TAP1-TAP2(E632Q) compared with

The Jackson Laboratory. Cells were incubated for 1 h with each antibody in phosphate-buffered saline, 1% fetal bovine serum, and the same buffer was used for washing cells. Flow cytometric analyses were performed using a BD Biosciences FACSCaliber cytometer and CellQuest software.

For analyzing TAP1 expression in SK-19 cells, cell lysates or microsome preparations were separated by SDS-PAGE, followed by immunoblotting analyses with purified anti-TAP1 (obtained from Dr. Soldano Ferrone). Microsome preparation protocols were similar for SK-19 cells, as described above for insect cells. For analyzing TAP2 expression in STF-1 cells, media was removed from confluent plates of STF-1 cells and replaced with methionine and cysteine-deficient Dulbecco’s modified Eagle’s medium (Invitrogen) containing 10% dialyzed fetal bovine serum (Invitrogen). The plates were further incubated at 37 °C for 30 min. Cells were trypsinized, centrifuged, and resuspended in 1 ml of methionine and cysteine-deficient Dulbecco’s modified Eagle’s medium. Cells were labeled with 0.2 μCi of [35S]methionine/cysteine (ICN) for 2 h. The cells were lysed with lysis buffer (1% Triton X-100, 10 mM Tris, 130 mM NaCl, complete EDTA-free protease inhibitors (Roche)) for 45 min before centrifuging for 20 min. Lysates were incubated for 45 min with 25 μl of 435.3 (21) supernatants or with anti-TAP2 antisera. 20 μl of protein G beads were added, and incubated at 4 °C for an additional 45 min. The beads were washed three times with lysis buffer. SDS-PAGE was added, and samples were boiled for 5 min. The immunoprecipitated proteins were separated by 10% SDS-PAGE gels. Gels were dried for 1 h and exposed for 24 h. A Typhoon phosphorimager (Amersham Biosciences) was used to visualize immunoprecipitated proteins. A faint background band that migrated at a similar position as TAP2 was visualized in immunoprecipitates of the parent STF-1 cells. This background band is not TAP2 as the truncated endogenous TAP2 expressed in STF-1 cells has an expected molecular mass of 30 kDa (stop codon follows residue 273 of TAP2 (25)), which should not be recognized by the 435.3 antibody or anti-TAP2 antisera that are directed against TAP2 NBD.
ATP Hydrolysis at the TAP1 and TAP2 Nucleotide Binding Sites

FIGURE 2. Peptide translocation by TAP complexes mutated at Asp⁶⁶⁸ of TAP1, Glu⁶³² of TAP2, or both. A–C, indicated microsomes (wild type (WT), mutant (M), or control microsomes (C) expressing a single subunit of TAP or lacking both subunits were used for peptide transport assays. The first bar in each pair corresponds to transport measured in the absence of ATP, and the second bar represents the transport measured in the presence of ATP. The figures show results from two representative sets of analyses for each mutant, and each bar is the average of triplicate measurements within an experiment. Error bars represent the S.E. ± means signals. The TAP1(D668N)-TAP2 complex is able to transport peptide (A), low to residual activity is observable with TAP1-TAP2(E632Q) (B), whereas the double mutant (C) did not display measurable activity by these assays. The WT microsomes used were TAP1-TAP2 for comparisons with TAP1-TAP2(E632Q), and TAP1-his-TAP2 for comparisons with TAP1(D668N)-TAP2 and TAP1(D668N)-TAP2(E632Q). D, immunoblots of WT and mutant (M) microsomes used in the translocation experiments of A–F, with TAP1- or TAP2-specific antibodies. TAP1 (left panels) and TAP2 (right panels) bands visualized just above the 61-kDa marker are indicated. Panels 1–2, 3–4, and 5–6 indicate TAP expression in WT and M microsomes used for the translocation experiments depicted in A–C, respectively. 1, 3, and 5 correspond to microsome preparations used in experiments shown in the left panels of A–C, and 2, 4, and 6 correspond to microsome preparations used in experiments shown in the right panels of A–C. E, the + ATP/– ATP ratios for control, wild type, and the indicated mutant microsomes averaged across multiple experiments undertaken in this study. DN indicates TAP1(D668N)-TAP2, EQ indicates TAP1-TAP2(E632Q), and DN.EQ indicates TAP1(D668N)-TAP2(E632Q). The graph indicates average + ATP/– ATP ratios from four independent translocation experiments for TAP1(D668N)-TAP2, five independent experiments for TAP1-TAP2(E632Q), and four independent experiments for TAP1(D668N)-TAP2(E632Q) (containing five independent comparisons of expression matched wild type and mutant). Statistical analyses were conducted on the log(1 + ATP/− ATP) values from all the replicates with a Welsh two-sample t test. In each of the tests of hypotheses, H0 is the null hypothesis that there is not a statistically significant difference in the means between the two groups being considered, against the alternative hypothesis HA, that one group has significantly greater or smaller values (as indicated) than the other. For the TAP1(D668N)-TAP2 analyses, H0: WT = M versus HA: WT > M, p value = 0.4091 (not significant). Additionally, for TAP1(D668N)-TAP2, H0: C = M versus HA: C < M, p value = 0.004547 (highly significant). For the TAP1-TAP2(E632Q), analyses, H0: WT = M versus HA: WT > M, p value = 0.0004465 (highly significant). Additionally, for TAP1-TAP2(E632Q), H0: C = M versus HA: C < M, p value = 0.01052 (significant). Additionally, for TAP1(D668N)-TAP2(E632Q), H0: C = M versus HA: C < M, p value = 0.07645 (not significant).

the corresponding wild type complexes (TAP1-his-TAP2 and TAP1-TAP2, respectively) revealed no difference in calculated affinities (data not shown).

Peptide translocation experiments were next performed by incubating a radiolabeled TAP substrate RR[¹²⁵I]YNASTEL and microsomes expressing wild type or mutant TAP complexes. Transport of the peptide into microsomes was assessed in the presence or absence of ATP. Because the peptide contains a glycosylation motif, translocated peptide can be quantified by γ-counting of peptide that bound to concanavalin A-Sepharose beads (Fig. 2). A TAP complex is considered to be competent for translocation if the radioactivity observed in samples incubated in the presence of ATP (+ ATP) is higher than the corresponding signals observed in the absence of ATP (− ATP) (the − ATP samples conditions as the background readings for each microsome preparation). Fig. 2, A–C, shows two representative peptide translocation experiments for each of the single mutants and for the double mutant, with each bar graph displaying the averages of triplicate measurements within an experiment. Each analysis of mutant translocation activity (M) included comparisons with expression matched wild type TAP-expressing microsomes (WT), and a negative control (C) with...
ATP Hydrolysis at the TAP1 and TAP2 Nucleotide Binding Sites

Efficiencies of Labeling of TAP Complexes with 8-Azido-\(\text{microsomes}, indicating low but measurable activity. Statistical had slightly higher TAP2(E632Q) mutant microsomes/H18528 complexes were significantly impaired relative to wild type TAP.

Fig. 2, A–C, are indicated in Fig. 2D (1 and 2 for Fig. 2A, 3 and 4 for Fig. 2B, and 5 and 6 for Fig. 2C).

The absolute magnitudes of the radioactivity signals varied significantly between different translocation experiments. This variation is to be expected, arising due to factors such as (i) differences in TAP expression levels, (ii) differences in microsome purity, (iii) different specific activities of the radiolabeled peptide, and (iv) age of the microsomes, and other factors. Despite this variation in the absolute magnitude of the signals between experiments, each translocation experiment conducted with wild type microsomes and TAP(D668N)/TAP2 indicated a significantly higher signal for the +atp condition compared with the −atp condition, whereas this was not the case with control microsomes (Fig. 2A). For TAP1-TAP2(E632Q), the +atp signals were only slightly higher compared with the −atp signals (Fig. 2B), whereas very similar +atp and −atp signals were observed with TAP1(D668N)-TAP2(E632Q). Fig. 2E shows the compiled average +atp/−atp ratios from four independent peptide translocation experiments with TAP1(D668N)-TAP2, five independent experiments with TAP1-TAP2(E632Q), and four independent experiments with TAP1(D668N)-TAP2(E632Q). The compiled data in Fig. 2E, as well as the individual experiments shown in Fig. 2, A–C, indicated that the TAP1(D668N)-TAP2 combination was significantly translocation active, whereas TAP1-TAP2(E632Q) and TAP1(D668N)-TAP2(E632Q) complexes were significantly impaired relative to wild type TAP.

Furthermore, the TAP1-TAP2(E632Q) mutant microsomes had slightly higher +atp/−atp ratios compared with control microsomes, indicating low but measurable activity. Statistical analyses with a two-sample t test verified these conclusions (Fig. 2E).

The TAP1(D668N) and TAP2(E632Q) Mutations Enhance Efficiencies of Labeling of TAP Complexes with 8-Azido-nucleotides—The E632Q and D668N mutations target putative γ-phosphate contact residues; these mutations are not expected to alter nucleotide binding affinities. To verify this expectation, the nucleotide binding properties of wild type and mutant TAP subunits and complexes were compared by photoaffinity labeling with 8-azido-[γ\(^{32}\)P]ATP or 8-azido-[α\(^{32}\)P]ADP, using previously established procedures (18). Insect cell microsomes expressing the desired TAP protein(s) were incubated for 15 min on ice with increasing concentrations of 8-azido-[γ\(^{32}\)P]ATP or 8-azido-[α\(^{32}\)P]ADP, followed by UV irradiation. Unbound nucleotides were removed by centrifugation, proteins were resolved by SDS-PAGE, and gels analyzed by phosphorimaging analyses. The extent of labeling was quantified using ImageQuant software.

The 8-azido-nucleotide labeling efficiencies were profoundly enhanced for membrane-associated TAP1(D668N) compared with wild type TAP1, both when expressed alone (data not shown), and when expressed as a complex with TAP2-eYFP (Fig. 3, A–E). Pairing TAP1 with TAP2-eYFP rather than TAP2 allowed for labeling of TAP1 versus TAP2 to be resolved, as previously described (18). Compared with TAP1, more efficient labeling of TAP1(D668N) was observed in binding analyses undertaken with both 8-azido-[γ\(^{32}\)P]ATP and 8-azido-[α\(^{32}\)P]ADP (Fig. 3, B and D). These differences were observed despite the considerably lower expression of TAP1 in the mutant complexes compared with wild type complexes (Fig. 3A). Some enhancement in TAP2-eYFP labeling was also observed when it was paired with TAP1(D668N) rather than wild type TAP1, although enhancement of the labeling efficiency of TAP1(D668N) itself was much more significant than the observed enhancement of labeling of TAP2-eYFP (Fig. 3, B and D).

\(K_D\) values were derived by fitting the intensity versus 8-azido-nucleotide concentrations data to a one-site binding model as previously described (18) (Fig. 3, C and E, and Table 1). The derived affinities for binding to 8-azido-ATP and 8-azido-ADP were similar for TAP1 compared with TAP1(D668N), both when expressed alone and when expressed as a complex (Table 1). Thus, as expected from structural data and predictions, the mutation did not affect the affinity of nucleotide binding to TAP. The latter result was confirmed using nucleotide-agarose bead experiments with detergent-extracted TAP complexes. In these analyses, no significant enhancement in nucleotide-agarose bead binding was induced by the TAP1(D668N) mutation (data not shown). It is presently unclear what mechanisms underlie the observed enhancement of labeling efficiencies of TAP1(D668N) with 8-azido-ATP and 8-azido-ADP, both when TAP1(D668N) is expressed alone, and when it is expressed in complex with TAP2-eYFP. Based on several reports that the counterpart E632Q mutation of ABC transporter NBDs stabilizes nucleotide-bound NBD dimers (for example, Ref. 28), it is possible that enhanced labeling of TAP1(D668N) reflects the stabilization of NBD interactions both in the presence of 8-azido-ATP and 8-azido-ADP. By contrast to full-length membrane-associated TAP1(D668N), it is of interest that the purified NBD of human TAP1(D668N) did not display enhanced labeling with 8-azido-nucleotides, and also did not migrate differently on a gel filtration column than TAP1 NBD (data not shown). It is possible that homodimeric TAP1-TAP1 NBD interactions are more favored in the context of the full-length human TAP1(D668N), and that stabilization of such interactions at least partly accounts for the observed enhancement in the labeling efficiency of single subunit TAP1(D668N) and of TAP1(D668N) in complex with TAP2-eYFP. Because a TAP1-TAP2 complex is required for peptide binding, stabilization of homodimeric TAP1-TAP1 NBD interactions are expected to be non-productive for a transport cycle.

TAP2 when expressed alone binds weakly to 8-azido-[γ\(^{32}\)P]ATP as previously described (18); the TAP2(E632Q) mutation did not enhance the 8-azido-ATP binding affinity. Low labeling signals were obtained for binding of both TAP2 and TAP2(E632Q) to 8-azido-[γ\(^{32}\)P]ATP, which were difficult to quantify (data not shown). When TAP2 or TAP2(E632Q) were expressed in combination with TAP1-eGFP, labeling of TAP2(E632Q) was more efficient than that of TAP2, in analyses with both 8-azido-ATP and 8-azido-ADP (Fig. 3, G and H). The TAP2(E632Q) mutation also enhanced the labeling efficiency
FIGURE 3. Labeling with 8-azido-nucleotides of TAP complexes with a single TAP1(D668N) or TAP2(E632Q) mutation. A, immunoblotting analyses of microsomes expressing TAP1-eYFP/TAP2-eYFP (WT) or TAP1(D668N)/TAP2-eYFP (M) with TAP1 (upper panel) or TAP2 (lower panel) specific antibodies. B and D, membranes were labeled following incubations of WT or M microsomes shown in A with the indicated concentrations of 8-azido-[γ-32P]ATP (B) or 8-azido-[α-32P]ADP (D). TAP bands were visualized following SDS-PAGE of the labeled samples and phosphorimaging analyses. Identical exposure times were used for comparisons of wild type and mutant microsomes. C and E, band intensities from B and D were quantified (using ImageQuant software) and plotted as a function of 8-azido-nucleotide concentrations. Data are representative of two independent sets of analyses.

F, immunoblotting analyses of microsomes expressing TAP1-eGFP/TAP2 (WT, lanes 1 and 3) or TAP1-eGFP/TAP2(E632Q) (M, lanes 2 and 4). G and I, microsomes shown in lanes 1 and 2 of F were used in 8-azido-[γ-32P]ATP labeling analyses and microsomes shown in lanes 3 and 4 of F were used in 8-azido-[α-32P]ADP labeling analyses. Membranes were labeled following incubations with the indicated concentrations of 8-azido-[γ-32P]ATP (G) or 8-azido-[α-32P]ADP (I). TAP bands were visualized following SDS-PAGE of the labeled samples and phosphorimaging analyses. Identical exposure times were used for comparisons of wild type and mutant microsomes. H and J, band intensities from G and I were quantified (using ImageQuant software) and plotted as a function of 8-azido-nucleotide concentrations. Data are representative of two independent sets of analyses. In B, D, G, and I, lanes C indicate labeling of control microsomes lacking TAP, with 2 μM 8-azido-nucleotide, and lanes M indicate radiolabeled molecular weight markers.
TABLE 1

Apparent affinities of indicated TAP constructs for 8-azido-[γ-32P]ATP and 8-azido-[α-32P]ADP when expressed as single subunits or in complex with the indicated partner subunit

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<thead>
<tr>
<th>Subunit for which K&lt;sup&gt;D&lt;/sup&gt; value is derived</th>
<th>Partner subunit</th>
<th>K&lt;sub&gt;xATP&lt;/sub&gt;</th>
<th>K&lt;sub&gt;xDADP&lt;/sub&gt;</th>
</tr>
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<tbody>
<tr>
<td>TAP1</td>
<td>None</td>
<td>2.5</td>
<td>1.8 ± 0.6</td>
</tr>
<tr>
<td>TAP1(D668N)</td>
<td>None</td>
<td>1.1 ± 0.6</td>
<td>2.2 ± 2.3</td>
</tr>
<tr>
<td>TAP1</td>
<td>TAP2-eYFP</td>
<td>2.6 ± 1.5</td>
<td>0.7 ± 0.5</td>
</tr>
<tr>
<td>TAP1(D668N)</td>
<td>TAP2-eYFP</td>
<td>1.7 ± 0.6</td>
<td>2.0 ± 0.04</td>
</tr>
<tr>
<td>TAP2</td>
<td>TAP1</td>
<td>1.6 ± 1.3</td>
<td>0.7 ± 0.6</td>
</tr>
<tr>
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<td>2.7 ± 0.6</td>
<td>2.2 ± 1.0</td>
</tr>
<tr>
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<td>None</td>
<td>ND</td>
<td>2.3 ± 1.1</td>
</tr>
<tr>
<td>TAP2(eQ632CQ)</td>
<td>None</td>
<td>ND</td>
<td>4.9 ± 0.1</td>
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<td>TAP2(eQ632CQ)</td>
<td>1.1 ± 1.0</td>
<td>1.3 ± 1.4</td>
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</tbody>
</table>

<sup>a</sup> ND, not determined.

of TAP1-eGFP in complex (Fig. 3, G and J), to an extent greater than or equal to the enhancement of labeling of TAP2(E632Q) itself (Fig. 3, H and J). The observation that the TAP2(E632Q) mutation enhances 8-azido-ATP labeling of TAP1 residues (cross-labeling, Ref. 18) is consistent with the possibility that this mutation stabilizes nucleotide-bound conformations of both TAP1 and TAP2, such as would be observed in a transition-state TAP1-TAP2 NBD dimer. On the other hand, the small effect of the TAP1(D668N) interaction on TAP2 labeling (compared with TAP1 labeling) may be indicative of a less critical role for the TAP1 nucleotide binding site in the maintenance of TAP1-TAP2 NBD dimers. Our previous 8-azido-ATP labeling analyses with TAP mutants deficient in nucleotide binding are consistent with this possibility (18). As expected from labeling analyses with the single mutants, labeling of TAP1(D668N)-TAP2(E632Q) was more efficient than that of TAP1-TAP2 (data not shown), although because fluorescence protein-tagged versions of mutant TAP1 or TAP2 were not available, it was not possible to resolve labeling of the individual subunits.

The TAP2(E632Q) and (E632Q/H661A) Mutations Significantly Impact the Ability of TAP2 to Induce MHC Class I Surface Expression in TAP2-deficient Cells, whereas Small Effects Are Observed with the Counterpart TAP1 Mutations (D668N and D668N/Q701A)—To extend the analyses of peptide translocation activity to assessments of the abilities of the TAP mutants to restore MHC class I surface expression in TAP-deficient human cells, retroviral constructs were generated that encoded TAP1, TAP1(D668N), TAP2, and TAP2(E632Q). TAP2-deficient STF-1 cells were infected with the viruses encoding wild type TAP2 or TAP2(E632Q). TAP2 expression was verified by immunoprecipitation analyses (Fig. 4A, inset), and MHC class I surface expression was compared using flow cytometric analyses with the W6/32 antibody. Consistent with the marked reduction in the peptide translocation activity of TAP complexes that was induced by the TAP2(E632Q) mutation, the mutant had a significantly reduced ability to restore MHC class I surface expression in TAP2-deficient cells (Fig. 4A). Over 10 independent flow cytometric analyses, STF-1 cells infected with viruses encoding TAP2(E632Q) displayed an average mean fluorescence ratio of 42% relative to that observed with cells that were infected with wild type TAP2 (Fig. 4G). The analyses included multiple independent infections of STF-1 cells with different preparations of wild type and mutant viruses. The average mean fluorescence ratio of the parent uninfected STF-1 cells was 23% relative to cells infected with the wild type TAP2-encoding virus; Fig. 4G), indicating low transport activity of TAP complexes containing TAP2(E632Q) (Fig. 4G).

TAP1-deficient SK-mel-19 (SK-19) cells (24) were infected with viruses encoding wild type TAP1 or TAP1(D668N). TAP1 expression was verified by immunoblotting analyses of microsome preparations from the cells (Fig. 4B, inset), and MHC class I surface expression was compared using flow cytometric analyses with the W6/32 antibody. The mean fluorescence values of cells expressing TAP1(D668N) was only slightly altered relative to that observed with cells expressing wild type TAP1 (Fig. 4B). Averaged over six independent flow cytometric analyses, the activity of TAP1(D668N) was 87% relative to wild type (Fig. 4H).

These analyses showed fairly good correlations between the results of the peptide translocation assays undertaken in insect cells, and TAP-mediated restoration of MHC class I surface expression in mammalian cells. The peptide translocation assays in insect cells, however, seemed to have lower sensitivity, as the low activity of the TAP2(E632Q) mutant complexes was more readily detectable using TAP activity assays that measured restoration of MHC class I surface expression. The mammalian cell-based assays were therefore used to assess the functional properties of additional mutants of TAP2 and TAP1.

The glutamic acid residue following the Walker B motif and the switch region histidine have been suggested to form a catalytic dyad in other ABC transporters (11). To additionally explore the effect of the switch region residues on TAP activity, we generated another retroviral construct encoding the TAP2(E632Q/H661A) double mutant, and assessed the ability of the mutant to restore MHC class I surface expression in TAP2-deficient cells. The mean fluorescence values of cells expressing TAP2(E632Q/H661A) ranged from 27 to 34% relative to that observed with cells expressing wild type TAP2 (six measurements), only slightly greater than that of the parent STF-1 cells that were TAP2-deficient (mean fluorescence 19–31% relative to wild type) (Fig. 4E, C and G). In each of six independent flow cytometric analyses, the TAP2(E632Q/H661A) double mutant was more significantly impaired than the TAP2(E632Q) single mutant. However, in each of the analyses, residual enhancement of MHC class I surface expression was consistently observable in cells expressing the TAP2(E632Q/H661A) double mutant compared with unin-
FIGURE 4. Flow cytometric analyses of cell surface MHC class I expression in TAP2-deficient (STF-1) cells or TAP1-deficient (SK-19) cells upon infection with viruses encoding wild type or mutant TAP1 and TAP2. Flow cytometric analyses of STF-1 and SK-19 cells with W6/32, a MHC class I specific antibody, followed by a fluorescently conjugated secondary antibody. A, C, and E, flow cytometric analyses of STF-1 cells expressing wild type TAP2 (WT) or the indicated TAP2 mutants. Insets show TAP2 protein expression in the indicated infected cells or the parent cells that lack TAP2 (UI). The indicated bands migrate close to the 66-kDa radiolabeled marker. The abbreviations are: WT for wild type TAP2, EQ for TAP2(E632Q), EQHA for TAP2(E632Q/H661A), ED for TAP2(E632D), HQ for TAP2(H661Q), and EDHQ for TAP2(E632D/H661Q). B, D, and F, flow cytometric analyses of SK-19 cells infected with viruses encoding wild type TAP1 (WT) or indicated TAP1 mutants. Insets show immunoblotting analyses of TAP1 protein expression in microsomes derived from cells expressing the indicated TAP1 constructs or from parent cells that lack TAP1 (UI). TAP1 bands visualized just above the 61-kDa bands are indicated. The abbreviations are: WT for wild type TAP1, DN for TAP1(D668N), DNQA for TAP1(D668N/Q701A), DEQH for TAP1(D668E/Q701H). G and H, average relative mean fluorescence values from cells expressing the indicated mutant constructs relative to that of the corresponding wild type TAP construct. Data are average of 16 (WT), 15 (None), 10 (EQ), 6 (EQHA), 4 (ED), 5 (HQ), and 2 (EDHQ) independent fluorescence measurements for TAP2 constructs and 8 (WT), 8 (None), 6 (DN), 4 (DNQA), and 6 (DEQH) independent fluorescence measurements for TAP1 constructs. For each comparison between wild type and mutant TAP, simultaneous virus preparations and cell infections were undertaken for wild type and mutant. Within individual experiments, mean fluorescence values obtained with mutant TAP subunits were normalized relative to those obtained from a parallel infection with the corresponding wild type TAP subunits (which was always set to 1). Each experiment also included a negative control (none) the mean fluorescence of which was also normalized relative to wild type cells.
fected cells (representative analysis is shown Fig. 4C), indicating that the peptide translocation activity of TAP complexes was not completely impaired by the TAP2(E632Q/H661A) double mutation.

In TAP1 sequences, the switch region histidine is modified to a glutamine. We generated another retroviral construct encoding the TAP1(D668N/Q701A) double mutant, and assessed the ability of this mutant, relative to wild type TAP1, to restore MHC class I surface expression in TAP1-deficient cells. The TAP1(D668N/Q701A) was very similar to wild type TAP1 in its ability to restore MHC class I surface expression in SK-19 cells (Fig. 4D). The average activity of the TAP1(D668N/Q701A) was 96% relative to that of wild type TAP1 in four independent analyses of three separate infections of SK-19 cells with viruses encoding TAP1 or TAP1(D668N/Q701A) (Fig. 4H). Thus, the additional Q701A mutation did not reduce the high peptide translocation activity of TAP complexes containing TAP1(D668N).

The TAP2(E632D) and (H632Q) Mutations Result in Attenuated TAP Activity, whereas the TAP1(D668E/Q701H) Mutation Induces a Slight Increase in TAP Activity—In TAP1 sequences, Asp$^{668}$ and Gln$^{701}$ replace highly conserved glutamic acid and histidine residues, respectively, in other ABC transporters (Fig. 1). Our results (Figs. 2 and 4) indicated that TAP1(D668N) and TAP1(D668N/Q701A) mutations affected TAP function less significantly than the counterpart TAP2(E632Q) and TAP2(E632Q/H661A) mutations. Together, these observations raised the possibility that the naturally occurring Asp$^{668}$ and Gln$^{701}$ alterations of TAP1 contributed to an attenuated, non-critical ATPase activity at the TAP1 site. To further examine this possibility, we analyzed the effects of TAP2(E632D), TAP2(H661Q), and TAP2(E632D/H661Q) mutants upon TAP activity in STF-1 cells. Indeed, each of the mutations significantly affected the ability of TAP to induce MHC class I surface expression (Fig. 4E). The TAP2(E632D) mutation (41% activity relative to wild type) affected TAP activity more significantly than the TAP2(H661Q) mutation (63% relative to wild type), and the double mutant (39% relative to wild type) exhibited a similar level of impairment as the TAP2(E632D) mutation. Each of the mutants displayed higher MHC class I surface expression compared with the parent uninfected cells (23% relative to wild type), indicating that the mutations reduced but did not abrogate TAP activity. We next examined the effects of restoring both Asp$^{668}$ and Gln$^{701}$ of TAP1 to the corresponding consensus residues found in other ABC transporters. In five independent flow cytometric analyses of SK-19 cells infected with viruses encoding wild type TAP1 or TAP1(D668E/Q701H), the mean fluorescence values of cells expressing TAP1(D668E/Q701H) ranged from 104 to 121% relative to that observed with cells expressing wild type TAP1 (Fig. 4, F and H). Based on these analyses, we concluded that mutations of catalytic site residues within the TAP1 nucleotide binding site had relatively small effects upon peptide translocation and MHC class I surface expression. Mutations away from consensus slightly decreased TAP activity, and mutations toward the consensus sequence slightly enhanced TAP activity, but the net increase or decrease induced by the alterations were relatively small, in the presence of a functional TAP2 catalytic site.

An important question that arises is whether the results shown in Fig. 4, A, C, E, and G, are directly comparable with those displayed in Fig. 4, B, D, F, and H, because the MHC haplotypes of the cell lines are likely to be different. Whereas MHC class I molecules vary greatly in their tapasin dependence, cell surface expression of nearly all well characterized MHC class I molecules is strongly impacted by TAP deficiency. Even HLA-B*4405 that assembles rapidly and efficiently, and that is highly tapasin independent for its cell surface expression, is highly dependent on TAP for its expression in SK-19 cells (29). Additionally class I molecules such as HLA-A2, which can bind to signal sequence-derived peptides, also display reduced surface expression in the absence of a functional TAP (30). The data displayed in Fig. 4, G and H, indicate relative MHC class I surface expression levels in cells expressing the indicated mutant TAP constructs compared with cells expressing the corresponding wild type TAP, or cells that lack one of the TAP subunits (none). The main conclusion we draw from this data are that none of the TAP1 mutations significantly impacted MHC class I surface expression, whereas all of the TAP2 mutations did significantly impact class I surface expression. Most human cells express six different class I allotypes. Whereas it is a formal possibility that the six class I molecules expressed by STF-1 cells are, on average, more susceptible to peptide depletion than those expressed by SK-19 cells, this seems unlikely because: 1) the mean fluorescence of uninfected SK-19 and STF-1 cells were both ~23% relative to that of the cells infected with TAP1 and TAP2, respectively. Thus the average surface expression of six different allotypes increases about 4-fold in both cell types in response to infections with viruses encoding the appropriate TAP subunit (even though the particular class I molecules responding in each cell type are likely to be different); thus both cells lines appear to be similarly affected by the introduction of TAP. 2) Expression of a different TAP1 mutant (TAP1(G646D)) markedly impacted TAP activity in SK-19 cells (29, 31). Thus, the averaged class I expression in SK-19 cells is indeed sensitive to the specific TAP1 expressed by the cells. However, the specific TAP1 mutants investigated in Fig. 4 did not appear to impact TAP1 activity.

**DISCUSSION**

Various modifications of TAP2 at Glu$^{632}$ and His$^{661}$, including E632D and H661Q, significantly reduced the catalytic activity of TAP complexes (Figs. 2 and 4, A, C, E, and G). The naturally occurring modifications of the counterpart residues of TAP1, Asp$^{668}$ and Gln$^{701}$, are likely to contribute to the natural attenuation of catalytic activity at the TAP1 site, as it has been very recently observed that the ATPase activity of isolated TAP1 NBD can be induced by the TAP1(D668E/Q701H) mutations (32). Consistent with this possibility, we observed that further modifications of these TAP1 residues away from the consensus sequence (D668N and D668N/Q701A) had only small effects upon peptide translocation and MHC class I surface expression (Figs. 2 and 4, B and D). It was of interest that restoration of the Asp$^{668}$ and Gln$^{701}$ to consensus sequences by the TAP1(D668E/Q701H) mutation only slightly increased
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TAP activity (Fig. 4F). If the TAP1(D668E/Q701H) mutation also enhances ATPase activity at the TAP1 nucleotide binding site of full-length TAP1-TAP2 complexes as was observed with TAP1 NBD (32), the results shown in Fig. 4F would indicate that TAP activity is not significantly enhanced by increasing ATPase activity at the TAP1 site. Together, these results suggest that, of the two nucleotide binding sites of TAP1-TAP2 complexes (Fig. 1), the TAP2 site is the main/essential active site required for peptide translocation and MHC class I surface expression. A non-essential role is indicated for ATPase activity at the TAP1 site. We and others have previously shown that exchanging the NBD of TAP1 and TAP2 resulted in “double chimeric” TAP complexes that were significantly active at the TAP1 site. We and others have previously shown that exchanging the NBD of TAP1 and TAP2 resulted in “double chimeric” TAP complexes that were significantly active for peptide transport (33, 34). Thus, although the TAP2 site is the critical active site required for peptide translocation by wild type TAP1-TAP2 complexes, this “active site” can be switched to the TAP1 site, with significant retention of catalytic activity.

The interpretations of the data presented here are admittedly incomplete in the absence of an ATPase assay for full-length TAP complexes. However, there is evidence from many other ABC transporters that a glutamic acid to glutamine mutation at the residue C-terminal to the Walker B motif, and modifications of the switch region histidine do impair ATP hydrolysis (6, 12–16). Although a peptide-stimulated ATPase activity has been described for TAP complexes partially purified and reconstituted from Raji (Human Burkett lymphoma) cells (35) (that express high levels of TAP complexes), direct demonstration of specific peptide-induced ATPase activity has been difficult with crude microsomal membranes expressing TAP complexes, or with TAP complexes purified from insect cell membrane (data not shown). We expect, based on the results described here, that steady state ATPase will be significantly reduced with all of the TAP2 mutants described here, but only slightly decreased (TAP1(D668N) and TAP1(D668N/Q701A)) or increased (TAP1(D668E/Q701H)) for the TAP1 mutants described here. Consistent with key roles for the Walker B glutamate and the switch region histidine residues in mediating ATP hydrolysis, using the isolated NBD domain of human TAP1, the results of Ernst et al. (32) showed that although wild type TAP1 NBD displayed no significant ATPase activity, the TAP1 NBD(D668E/Q701H) double mutant had ATPase activity. Additionally, the TAP1 NBD(Q701H) single mutant had ATPase activity, whereas TAP1 NBD(D668E) showed no significant ATPase activity. These data indicated a more important role for the switch region histidine in mediating ATP hydrolysis within isolated TAP1 NBD; however, at the TAP2 site of full-length TAP complexes, it appears that the TAP2(E632D) mutation causes a slightly greater loss in TAP activity compared with the TAP2(H661Q) mutation (Fig. 4E). Whereas more studies will be needed to better understand the precise contributions of the glutamic acid and histidine residues in mediating the hydrolytic event, the ATPase activity results with TAP1 NBD(D668E/Q701H) compared with TAP1 NBD (32) support our conclusions from Figs. 2 and 4 that the presence of AspGln and GlnH101 indeed contribute to the natural attenuation of the catalytic activity of the TAP1 site, and that ATP hydrolysis at the TAP1 site is not critical for the TAP transport cycle.

A relevant question that follows from these studies is whether the TAP1 site is capable of ATP hydrolysis at all. Other studies that used trapping of 8-azido-nucleotides onto TAP in the presence of phosphate transition state analogs have suggested that ATP hydrolysis occurs at both sites in TAP1-TAP2 complexes, because nucleotide trapping was observed on both TAP1 and TAP2 (36). However, these results were based on the interpretation that 8-azido-nucleotide trapping on TAP1 resulted from hydrolysis at the TAP1 site, and that 8-azido-nucleotide trapping on TAP2 resulted from hydrolysis at the TAP2 site. In a model of TAP1-TAP2 based upon the MJ0796 structure (6), the C8 carbon of a TAP2-bound nucleotide is predicted to be in close proximity to Tyr477 of TAP2 as well as Gln642 of TAP1 (Fig. 1B). Because cross-linking by an azido group is not residue-specific, an 8-azido-ATP bound at the TAP2 site could potentially be cross-linked in the vicinity of either of these residues, which could result in some degree of TAP1 labeling by nucleotide bound at the TAP2 nucleotide binding site. Alternatively, blocking hydrolysis at a single site, such as with the TAP2(E632Q) mutant described here, could result in enhanced labeling of both TAP1 and TAP2, if the complexes are trapped in a dimeric conformation (Fig. 3f). Thus, 8-azido-ATP trapping analyses may be reflective of steady state ATPase activity rather than that occurring at the individual nucleotide binding sites.

Based on studies with TAP chimeras, we previously suggested that both TAP nucleotide-binding domains were catalytically active, although to different extents. Chimeric human TAP2 in which the NBD of TAP2 was replaced with that from human TAP1 (T2MT1C; containing a TAP2 membrane-spanning domain and a TAP1 nucleotide-binding domain) was competent for peptide translocation when paired with TAP1 (TAP1-T2MT1C complexes) (34), but with reduced efficiency compared with wild type TAP1-TAP2 complexes. Because the chimeric TAP1-T2MT1C complexes contained non-consensus residues (AspGlnt and GlnH101) at both nucleotide binding sites, the naturally occurring GluAsp and HisGln alterations at these residues apparently did not completely abrogate the ability of the TAP1 site to hydrolyze ATP (consistent with the low but measurable activity of TAP2(E632D/H661Q); Fig. 4, E and G). Analysis of the function of the TAP1-T2MT1C chimeric complex did not, however, reflect on the ATPase activity of a true TAP1 nucleotide binding site, because a TAP1 nucleotide binding site in TAP1-TAP2 complexes has an LAAGQKQR sequence in the vicinity of the signature region (rather than the counterpart LSGGQKQR sequences of the TAP1-T2MT1C complexes) (Fig. 1). In TAP1-TAP2 complexes, these alterations might further hinder NBD interactions and ATPase activity at the TAP1 nucleotide binding site. Nevertheless, the present findings also support the possibility of a low ATPase activity at the TAP1 nucleotide binding site. Although both GluAsp and HisGln of TAP2 are predicted to be critical for the catalytic activity based on models that invoke general base-type catalysis (6) or substrate-assisted catalysis (11), respectively, none of the mutants described here completely destroyed TAP activity. When the ATPase activity of the TAP2 site is reduced to a level below that of the TAP1 site (as might be the case with TAP2(E632Q/H661A)), it is possible that hydrolysis at the TAP1 site drives the residual transport.
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FIGURE 5. Model for the catalytic cycle of TAP complexes. In resting state TAP complexes (1), NBD of TAP1 and TAP2 are loosely engaged. Peptide binding to TAP1-TAP2 complexes promotes ATP binding to the TAP2 site (1 → 2), and conformational changes that facilitate tight TAP1 NBD-TAP2 NBD interactions (2 → 3). This step may be followed by a reorientation of the peptide binding site toward an opening in the membrane, accompanied by a reduction in the peptide binding affinity, which results in peptide release into the ER lumen. ATP hydrolysis then occurs at the TAP2 site (3 → 4), which is impaired by several TAP2 mutants described here. The hydrolysis step could drive further conformational rearrangements at the NBD-NBD interface, which are transmitted to the transmembrane domains, to restore a peptide binding site on the cytosolic surface and reinitiate a new cycle (4 → 1). ATP hydrolysis at the TAP1 site (4 → 5) may allow for more efficient disengagement of NBD dimers (5 → 6), and reset to 1. Based on the minor effects of the TAP1 mutants described here upon TAP activity, 5 and 6 appear largely dispensable to the TAP catalytic cycle.

Whereas TAP1 nucleotide binding site mutants described in this study had only small or no effects on peptide transport, several previously described TAP1 nucleotide binding site mutants of residues within the Walker A motif of TAP1 (17, 37–39), or mutants within TAP2 signature motif residues (40, 41), were completely impaired or had only residual transport activities. Thus, although ATPase activity at the TAP1 nucleotide binding site does not appear to be critical for TAP activity, the TAP1 nucleotide binding site, nevertheless, plays an important role in the transport cycle, likely in the initial steps leading to the formation of the active transition state TAP1-NBD-TAP2-NBD dimer. Fig. 5 depicts a working model for the catalytic cycle of TAP, integrating information from the present analyses, previous biochemical studies of TAP complexes (17, 18, 35, 37–41), as well as structural studies of ABC transporters (4–10). The essential catalytic steps of the TAP transport cycle are outlined in steps 1–4. The resting state of TAP complexes may involve a conformation such as that depicted in step 1 in which TAP1 and TAP2 NBD are loosely engaged, with TAP1 in an ATP bound conformation, and TAP2 in an ADP bound conformation. Binding of peptide may induce a conformational change that enhances ATP binding at the TAP2 site and promotes tight NBD interaction across the TAP1-TAP2 Walker A/B-signature motif interfaces (steps 2 → 3). These conformational rearrangements at the interface of the NBD (steps 2 → 3) could in turn induce a reorientation of the substrate binding site toward the side facing the ER, accompanied by a reduction in the affinity of the substrate binding site, as previously suggested (42). ATP hydrolysis at just the TAP2 site could then drive further conformational rearrangements at the NBD-NBD interface, that are transmitted to the transmembrane domains, to restore a peptide binding site on the cytosolic surface and reinitiate a new cycle (step 4 → step 1). Alternatively, step 4 of the TAP catalytic cycle could be followed by a slower step of ATP hydrolysis at the TAP1 nucleotide binding site (step 4 → 5), which would allow for more reset (step 5 → 6), and an overall enhancement in the transport rate. The minor functional consequences of the TAP1 mutations indicate that steps 5–6, if occurring in the TAP transport cycle, are largely dispensable.

The model proposed in Fig. 5 is similar to the processive clamp model proposed for ABC transporters by van der Does and Tampé (43), and also the model proposed by Reits et al. (44) for the TAP transport cycle, but establishes a critical role for ATP hydrolysis at the TAP2 site in maintaining the transport cycle. Furthermore, based on the cystic fibrosis transmembrane conductance regulator model (45) and the van Endert et al. (2) model proposed sequential ATP interactions with the TAP2 site first, followed by interaction with the TAP1 site, whereas Fig. 5 incorporates a transition state NBD dimer enclosing two ATP molecules. The existence of such a dimer would be consistent with several structural studies (4–6, 9), as well as biochemical data that showed that when TAP1 and TAP2 were in complex, 8-azido-nucleotides labeled both subunits with similar affinities (18). The working model in Fig. 5 provides a template for further mechanistic investigations of the TAP transport cycle, and several aspects of this model need experimental clarification.

A mechanistic model involving one main active site (that with the faster catalytic rate) is likely to apply to other ABC transporters that have an asymmetric distribution of catalytic site residues. These include ABCB1–6 and ABCB10–11 (multidrug resistance proteins), ABCB8 and ABCB9 (SUR1 and SUR2) that all contain Walker B Glu→Asp substitutions in their NBD1, and NBD1 of ABCB7 (cystic fibrosis transmembrane conductance regulator, which has a Walker B Glu→Ser substitution). In ABCB11 (bile salt export pump), a Walker B Glu→Met substitution is observed, and in yeast pleiotropic drug resistance protein (Pdr5p), a Walker B Glu→Asn substitution is observed. In cystic fibrosis transmembrane conductance regulator NBD1, a serine replaces the conserved histidine in the switch region, and NBD1 of ABCA6 and ABCA8→10 all contain a glutamine in place of a histidine in the...
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switch region. All of these transporters are likely to have attenuated ATPase activity in one of their nucleotide binding sites, resulting in their second site being the main catalytic site driving peptide transport. For other ABC transporters such as P-glycoprotein that lack an obvious asymmetric distribution of catalytic site residues or structural asymmetry, hydrolysis could likely be initiated at either of the two catalytic sites. The requirement for a second hydrolysis step, and the extent of catalytic site coupling, might be variable between the different transporters, and determined by the type and extent of interactions occurring at particular NBD-NBD interfaces.

In summary, the studies described here support a mechanism in which the main active site required for peptide translocation by TAP1-TAP2 complexes resides at the TAP2 nucleotide binding site. Key catalytic residue modifications at the TAP1 site are predicted to reduce the catalytic activity at this site as recently shown with the NBD of TAP1 (32), and ATPase activity at the TAP2 site appears to be largely sufficient to drive peptide transport. These results may seem surprising in light of our previous observations that chimeric TAP complexes with two TAP2 NBDs (TAP2 paired with T1MT2C, a TAP chimera containing the TAP1 membrane spanning domain and a TAP2 nucleotide-binding domain) were significantly less active (rather than more active) than chimeric TAP complexes containing two TAP1 NBDs (TAP1/T2MT1C) (34). However, it is important to note that for high affinity nucleotide binding, the TAP2 nucleotide binding site critically depends upon the presence of a TAP1 NBD. Chimeric TAP complexes with two TAP2 NBDs bound nucleotide-agarose beads and 8-azido-nucleotides with significantly reduced affinities compared with wild type TAP complexes or TAP complexes with two TAP1 NBDs (18), and likewise single subunit TAP2 has a low affinity nucleotide binding site in the absence of TAP1 (Ref. 18 and Table I). These observations indicate that even though many of the catalytically active residues reside on TAP2, TAP1 NBD is critical for optimal nucleotide binding to the TAP2 site, and for initiating the early steps of the TAP catalytic cycle (Fig. 5, steps 1–3).

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Note Added in Proof—Findings relevant to this study have recently been published (46).

REFERENCES
ATP Hydrolysis at the TAP1 and TAP2 Nucleotide Binding Sites

Catalytic Site Modifications of TAP1 and TAP2 and Their Functional Consequences
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