Walker A Lysine Mutations of TAP1 and TAP2 Interfere with Peptide Translocation but Not Peptide Binding*

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We generated mutants of the transporter associated with antigen-processing subunits TAP1 and TAP2 that were altered at the conserved lysine residue in the Walker A motifs of the nucleotide binding domains (NBD). In other ATP binding cassette transporters, mutations of the lysine have been shown to reduce or abrogate the ATP hydrolysis activity and in some cases impair nucleotide binding. Mutants TAP1(K544M) and TAP2(K509M) were expressed in insect cells, and the effects of the mutations on nucleotide binding, peptide binding, and peptide translocation were assessed. The mutant TAP1 subunit is significantly impaired for nucleotide binding relative to wild type TAP1. The identical mutation in TAP2 does not significantly impair nucleotide binding relative to wild type TAP2. Using fluorescence quenching assays to measure the binding of fluorescent peptides, we show that both mutants, in combination with their wild type partners, can bind peptides. Since the mutant TAP1 is significantly impaired for nucleotide binding, these results indicate that nucleotide binding to TAP1 is not a requirement for peptide binding to TAP complexes. Peptide translocation is undetectable for TAP1-TAP2(K509M) complexes, but low levels of translocation are detectable with TAP1(K544M)-TAP2 complexes. These results suggest an impairment in nucleotide hydrolysis by TAP complexes containing either mutant TAP subunit and indicate that the presence of one intact TAP NBD is insufficient for efficient catalysis of peptide translocation. Together, these results also suggest the possibility of distinct functions for TAP1 and TAP2 NBD during a single translocation cycle.

The transporter associated with antigen processing (TAP) is a critical component of the major histocompatibility complex (MHC) class I antigen presentation (1–3). TAP functions to translocate peptides from the cytosol to the ER. Binding of peptides to newly synthesized MHC class I molecules in the ER stabilizes the MHC class I heterodimer and allows transit of MHC class I-peptide complexes to the cell surface for immune surveillance by T cells (4). Two structurally related subunits of the TAP transporter, TAP1 and TAP2, form a complex on the ER membrane that is necessary and sufficient for peptide translocation from the cytosol into the ER. The cytosolic face of TAP1-TAP2 complexes contains a binding site for peptides (5), which can function in the sequestration of peptides derived from proteasomal proteolysis. A recently discovered protein called tapasin is associated with the TAP1-TAP2 complex (6, 7). Tapasin has been shown to enhance the expression level of TAP1 and increase peptide transport by TAP complexes (8). However, tapasin is not required for peptide binding by TAP1-TAP2 complexes or for translocation per se, since TAP1-TAP2 complexes expressed heterologously in insect cells can bind and transport peptides (9).

TAP is a member of the ATP-binding cassette (ABC) family of transmembrane transport proteins (10). Members of this family transport various substrates across cellular membranes in an ATP-dependent manner. Known substrates of ABC transporters include amino acids, peptides, proteins, sugars, and lipids. The typical ABC transporter has two hydrophobic membrane-spanning regions with multiple membrane-spanning segments and two cytosolic nucleotide binding domains (NBD). The NBD contains several conserved sequence motifs. These include the Walker A and Walker B sequence motifs, which are characteristic of nucleotide binding folds (11). The membrane-spanning segments and the NBD can occur in a single polypeptide as in the mammalian cystic fibrosis transmembrane conductance regulator and P-glycoprotein, as two separate polypeptides as in the TAP transporters, or as four separate polypeptides as in many bacterial ABC transporters (10).

TAP1 and TAP2 each comprise one membrane-spanning region with several membrane-spanning segments and one cytosolic NBD. In vitro experiments with intact TAP domains as well as with each truncated NBD have shown that both TAP1 and TAP2 bind ATP and ADP (12–15). Indirect evidence for the occurrence of ATP hydrolysis during translocation comes from observations that the addition of exogenous ATP is required for peptide translocation by TAP and that translocation is not supported by nonhydrolyzable ATP analogs or ADP (16). However, the catalysis of ATP hydrolysis by TAP complexes or either NBD remains to be directly demonstrated. It is not known whether ATP hydrolysis by TAP1, TAP2, or the complex is required for a completion of a catalytic cycle. Furthermore, the requirement for nucleotide binding to each TAP subunit upon substrate (peptide) interactions with the TAP complex also remains to be unambiguously defined. Peptide binding to TAP was initially suggested to be nucleotide binding-independent, based upon studies of peptide binding to microsomal membranes expressing wild type TAP1 and TAP2 in the presence or absence of apyrase, an enzyme that depletes ATP and ADP (5). However, based upon more recent studies using TAP1 and

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‡ The abbreviations used are: TAP, transporter associated with antigen processing; MHC, major histocompatibility complex; ABC, ATP-binding cassette; NBD, nucleotide binding domain(s); PBS, phosphate-buffered saline; HPLC, high pressure liquid chromatography.

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TAP2 mutants that both lacked nucleotide binding activity, it was suggested that peptide binding by TAP is dependent upon nucleotide interactions with one or both subunits of the TAP1-TAP2 complex (17). Further experiments are required to resolve this discrepancy and to establish whether nucleotide binding to TAP1, TAP2, or both subunits alters the affinity of TAP1-TAP2 complexes for peptides.

Toward a definition of the requirement for nucleotide binding and hydrolysis by each TAP subunit for peptide binding and translocation, we generated mutants of TAP1 (K544M) and TAP2 (K509M) that were altered at a conserved lysine residue of the Walker A motif (GXXGXGK/S/T) of each protein. Based upon the crystal structure of the ATP binding subunit of histidine permease, a bacterial ABC transporter, the Walker A lysines of TAP1 and TAP2 are predicted to be located on the phosphate binding loop (P-loop) and form contacts with the β-phosphate of ATP (18). Mutations of this lysine in other ABC transporters have been shown to impair nucleotide binding and/or hydrolysis and impair substrate translocation (19–23). We observe that the Walker A lysine mutations in TAP1 and TAP2 have distinct effects upon nucleotide binding to each subunit, with nucleotide binding being significantly impaired in the TAP1(K544M) mutant but not in the TAP2(K509M) mutant. The observation that the TAP1(K544M) mutation is significantly impaired for nucleotide binding by TAP1 also allowed us to determine the correlation between nucleotide binding by TAP1 and peptide binding to TAP1-TAP2 complexes. Additionally, assays of peptide translocation by each mutant allowed insights into the requirement for nucleotide hydrolysis by TAP2 during a translocation cycle. Taken together, these results allow for the refinement of a model for the mechanistic steps involved during a single peptide translocation cycle.

**Experimental Procedures**

**Construction of Baculoviruses Encoding Mutant TAP Subunits**—The cDNAs for TAP1 and TAP2 were obtained from Dr. John Trowsdale. Polymerase chain reaction was used to introduce a sequence encoding a His tag to the 5′-end of TAP1 for 2 h at 72 °C. The modified cDNAs were cloned into pPCR2.1. Site-directed mutagenesis was performed by the standard procedure (9). 30 μg of insect cell microsomes containing wild type or mutant TAP1-TAP2 complexes or single subunits were added to 150 μl of assay buffer (PBS, 0.1% bovine serum albumin, 1 mM dithiothreitol, pH 7.3) containing 10 mM MgCl2 and 5 mM ATP (ATP samples) or 0.03 units/μl of apyrase (ATP) (Sigma). Radioiodinated RYQKCTEL peptide was then added, and the samples were incubated at 37 °C for 15 min. The samples were then centrifuged at 4 °C at 8,500 × g and washed once with 250 μl of the appropriate assay buffer. The pellets were then resuspended in 250 μl of lysis buffer (50 mM Tris, 150 mM NaCl, 1% Nonidet P-40, pH 7.4) and incubated on ice for 1 h. After centrifugation at 8,800 × g for 5 min at 4 °C, the supernatants were transferred to ConA-Sepharose (Amersham Pharmacia Biotech) beads and washed with Tris-buffered saline and a second round of Tris-buffered saline containing 1 M NaCl. The modified peptide was incubated with the appropriate assay buffer, and the radioactivity was determined using a Beckman 5500 γ-counter. Results from microsome-based translocation assays were verified using SF21 cell-based assays, as per previously described protocols (26).

**Peptide Binding Assay**—Peptide binding assays were carried out using a recently described protocol (27). All peptides were synthesized using automated Merrifield solid-phase synthesis in a CH3CN-NH2 solvent mixture. For the binding experiments, a peptide was synthesized with the sequence RYQKCTEL and purified by reverse-phase HPLC. Peptide concentrations were normalized between experiments by measuring the absorbance at 225 nm (A225 = 0.28 for 0.01 mg/ml peptide).

**Fluorescence Quenching Assay**—The fluorescence emission signal ($F_{555} - F_{495}$) was recorded using a PTI fluorimeter equipped with a microstirring device. A 5-mm round quartz cuvette was filled with 180 μl of assay buffer (PBS, 1 mM dithiothreitol, 5 mM MgCl2, pH 7.4) at room temperature, and the fluorescent peptide RYQKCTEL was added with vigorous stirring (2.5–80 nM final concentration). The fluorescence signal was then allowed to stabilize. 20 μl of insect cell microsomes treated with 0.03 units/μl of apyrase or with 1 mM ADP or ATP (for at least 30 min on ice) were added to the cuvette. The decrease in fluorescence due to the decrease in fluorescence was monitored to nitromine was monitored. The time dependence of quenching was determined by fitting the data to an exponential function, $F(t) = F_0 - ΔF e^{-kt}$ (where $F_0$ is the fluorescence value at steady state, $ΔF$ is the net change in fluorescence, and $k$ is the rate constant) using nonlinear least squares analysis in the Prism software package (Graph Pad software). The total fluorescence quenching signal ($ΔF$) was plotted as a function of peptide concentration. The
curve was fitted to the equation $y = \Delta F + \frac{[P]}{K_d + [P]}$, and $K_d$ values were determined by nonlinear least squares analysis.

After stabilization of fluorescence signals, unlabeled peptide was added at a concentration of ~30 μM, and the fluorescence recovery was monitored over several seconds as bound fluorescent peptide dissociated. The dissociation rate constants, $k_d$, were estimated by fitting the fluorescence recovery signal to a monoexponential function, $y = F_0 + \Delta F + e^{-t/k_d}$ (where $F_0$ is the end point fluorescence value, $\Delta F$ is the net change in fluorescence, and $k_d$ is the dissociation rate constant).

Each set of binding experiments included a control with microsomal membranes prepared from uninfected Sf21 cells. Fluorescence quenching signals were also sometimes observed with microsomal membranes prepared from infected cells, but recovery was typically not observed (Figs. 4 and 5). At peptide concentrations below 80 nM, the quenching signals observed for the control microsome preparations (if any) were typically significantly lower than those observed with microsome preparations containing wild type or mutant TAP complexes (Fig. 5, A–C). Variations in the quenching signal observed for microsome preparations from uninfected cells (ranging from negative amplitudes to low positive amplitudes as shown in Fig. 5, A–C) might arise due to peptide degradation during storage. In general, the lowest background signals were observed for experiments conducted within 1 week of HPLC purification of peptide, when the purified peptide was stored at ~20 °C under dessication.

RESULTS

Expression and ATP Binding by TAP1 and TAP2 Mutants—We modified the TAP1 cDNA by polymerase chain reaction, to introduce a sequence encoding a C-terminal hexahistidine tag (TAP1-His), to facilitate the biochemical analyses of TAP1 expression and function. The TAP1-His protein sequence was further modified by site-directed mutagenesis (24), in order to alter a conserved lysine residue in the Walker A motif (Lys$^{544}$) to a methionine. A similar modification was introduced into the cDNA sequence encoding TAP2, in order to alter the Walker A lysine (Lys$^{509}$) to a methionine. Mutant clones were sequenced and ligated into the baculovirus transfer vector pAcUW51. Recombinant baculoviruses were generated encoding histidine-tagged TAP1 (TAP1-His), the corresponding Walker A lysine mutant (TAP1(K544M)-His), and the TAP2 Walker A lysine mutant (TAP2(K509M)). The anti-His antibody (Covance Scientific) was used to screen for TAP1 expression, and the TAP2-specific antibody 435.3 (anti-TAP2) (25) was used to screen for TAP2 expression. Baculoviruses encoding wild type TAP1 (TAP1) and TAP2 (TAP2) were obtained from the laboratory of Dr. Robert Tampé (9). For comparisons of nucleotide binding by each mutant or wild type TAP subunit, insect cells were infected with baculoviruses encoding wild type or mutant TAP complexes, insect cells were infected with the radiolabeled peptide in the presence or absence of ATP, ADP, or AMP-Sepharose beads for 2 h. Subsequently, the beads were washed, and proteins were eluted from the beads by boiling in the presence of SDS-polyacrylamide gel electrophoresis buffer. Proteins associated with the beads were analyzed by SDS-polyacrylamide gel electrophoresis and immunoblotting with anti-His or anti-TAP2 antibodies (Fig. 1). Lysates were also immunoprecipitated with anti-His or anti-TAP2 antibodies to directly visualize the relative expression levels of mutant versus wild type proteins. At comparable expression levels of TAP1-His and TAP1(K544M)-His (Fig. 1A, lanes 4 and 8), strong binding of TAP1-His to ATP-Sepharose and ADP-Sepharose beads is visualized (Fig. 1A, lanes 1 and 2), whereas TAP1(K544M)-His binding to ATP and ADP beads is barely detectable (Fig. 1A, lanes 5 and 6). These observations indicate that the K544M mutation impairs nucleotide binding to TAP1. By contrast, TAP2(K509M) binding to ATP and ADP beads does not appear to be significantly impaired relative to wild type TAP2 (Fig. 1B, lanes 1 and 2 compared with lanes 5 and 6).

Subunit Association and Peptide Translocation by Wild Type and Mutant TAP Complexes—It has previously been shown that TAP1 and TAP2 associate into stable complexes and that both subunits are required for peptide binding and translocation (28, 29). Prior to analysis of the peptide binding and translocation properties of the mutants, it was therefore necessary to demonstrate that each mutant could form TAP1-TAP2 complexes. To assay the formation of TAP1(K544M)-His/TAP2 complexes, cells were infected with viruses encoding TAP1(K544M)-His and TAP2, or with TAP1-His and TAP2 as the positive controls. Detergent lysates were immunoprecipitated with either anti-His or anti-TAP2 antibodies. The samples were subsequently separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes, and the membranes were stained with anti-His or anti-TAP2. We observed that TAP1(K544M)-His associates with TAP2 as does TAP1-His (Fig. 2A). Likewise, TAP2(K509M) associates with TAP1 as does wild type TAP2, as measured by immunoprecipitation analyses with the TAP1-specific antibody 148.3 (anti-TAP1) (9) and anti-TAP2 (Fig. 2B).

Peptide translocation experiments were carried out as previously described (9, 26). The model substrate RRYNASTEL was used, which was 125I-labeled. Microsomal membrane preparations containing wild type or mutant TAP complexes were incubated with the radiolabeled peptide in the presence or absence of ATP for 15 min at 37 °C. Since the peptide sequence contains an NAS glycosylation motif, translocated peptide was retained in the ER rather than being exported (30). Free peptide was separated by centrifugation, the microsomal membranes were lysed in detergent, and glycosylated peptide was quantified by determining their binding to ConA-Sepharose beads. The experiments were carried out with microsome preparations expressing wild type and mutant TAP1-TAP2 complexes. Microsome preparations expressing single TAP subunits were used as negative controls, since it has previously been shown that microsome preparations expressing wild type TAP1 alone or wild type TAP2 alone do not translocate peptides (9, 25). The results of a representative experiment are indicated in Fig. 3. Within an experiment, we defined a translocation signal as positive when the average cpm $\times$ ATP/cpm $\times$ ATP ratio was at least 2-fold above the ratio observed for the single subunit control. By this criterion, results from three independent translocation experiments indicated that TAP1-TAP2-
Peptide Binding by Wild Type and Mutant TAP Complexes—A fluorescence quenching assay has recently been described for quantitation of peptide binding to TAP complexes, under nucleotide-depleting conditions (27). We used these assays to quantify peptide binding by wild type and mutant TAP complexes. For this purpose, the peptide RRYQKCTEL was fluorescein-labeled using 5-iodoacetamidofluorescein and HPLC-purified. Microsomal membrane preparations containing each mutant TAP complex, the corresponding wild type TAP complexes, or neither TAP subunit (uninfected microsomes) were added to different concentrations of the fluorescent peptide, and the fluorescence emission signal was monitored. For the wild type as well as for the mutant TAP complexes, increased fluorescence quenching was observed as the peptide concentration was increased (Fig. 4, A–C). After stabilization of fluorescence signals, unlabeled peptide was added at a concentration of ~30 μM, and the fluorescence recovery was monitored over several seconds as bound fluorescent peptide dissociated. As for wild type TAP complexes, fluorescence recovery was also observed for both TAP mutants, and the magnitude of the recovery was in proportion to the magnitude of the quenching. The magnitude of the fluorescence quenching and recovery signals obtained for wild type and mutant TAP complexes were critically dependent upon the expression levels of both TAP subunits. In the experiments shown in Fig. 4, A and C, the same microsome preparations of TAP1-TAP2 and TAP1-TAP2(K509M) were used as for the translocation assays shown in Fig. 3 (Fig. 3B, lanes 1 and 2). At lower expression levels, reliable fluorescence quenching and
recovery was not observed. For example, with microsomal preparations of TAP1-His TAP2 (Fig. 3B, lane 3), fluorescence recovery was not observed upon the addition of excess unlabeled peptide. Increasing the expression level of both TAP1-His and TAP2, however, resulted in binding profiles that resembled wild type TAP1 TAP2 complexes (data not shown).

To verify the TAP dependence of the fluorescence quenching and recovery signals shown in Fig. 4, A–C, changes in fluorescence were also monitored upon the addition of excess unlabeled peptide. Increasing the expression level of both TAP1-His and TAP2, however, resulted in binding profiles that resembled wild type TAP1-TAP2 complexes (data not shown).

The steady state fluorescence quenching was determined from an exponential fit of the association data (averaged over two independent experiments for the same microsome preparation). The steady state fluorescence quenching values are plotted as a function of peptide concentration for microsomes containing wild type and mutant TAP complexes (Figs. 5, A–C). Each experiment summarized in Fig. 5, A–C, included a negative control with uninfected microsomes (also averaged over two independent experiments for the same microsome preparation). For most of the data points shown in Fig. 5, the amplitudes of the fluorescence quenching signals obtained with microsomes containing wild type or mutant TAP complexes were significantly greater than that obtained for microsomes from uninfected cells, over the indicated concentration range. Taken together with the fluorescence recovery data, we infer from these results that both sets of mutant TAP complexes are
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affinity of this mutant was slightly weaker compared with wild type TAP complexes. The observations that this mutant TAP complex can bind peptides (Figs. 4C and 5C) and nucleotides (Fig. 1B) but does not translocate peptides (Fig. 3A) indicate that the mutant is arrested at a stage that follows peptide binding and nucleotide binding, most likely at a step that requires nucleotide hydrolysis.

The calculated binding and rate constants for peptide interactions with TAP1-TAP2 complexes and TAP1-His–TAP2 are very similar to each other and to TAP1(K544M)-His–TAP2 complexes (Fig. 4, A and B, and Fig. 5, A and B; data not shown for TAP-His–TAP2 complexes). As mentioned above, the peptide binding experiments shown in Figs. 4 and 5 were carried out in the presence of apyrase (ATP and ADP-depleting conditions). It is a formal possibility that TAP-associated nucleotides cannot be accessed by apyrase and that one or both TAP subunits in wild type TAP complexes remain nucleotide-occupied in the presence of apyrase. However, since TAP1(K544M)-His is significantly impaired for nucleotide binding (Fig. 1), we expect that in the presence of apyrase, the nucleotide occupancy will be low for the TAP1(K544M)-His mutant in TAP1(K544M)-His–TAP2 complexes. The observation that the calculated binding constants and dissociation rate constants for TAP1(K544M)-His complexes do not differ significantly from the values derived for wild type TAP complexes when apyrase is present or when ADP is present (Table I) indicates that nucleotide interaction with TAP1 is not a requirement for peptide interactions with TAP1-TAP2 complexes.

**DISCUSSION**

It has previously been observed that the isolated nucleotide binding domains of TAP1 and TAP2 are capable of binding nucleotide (9, 13, 15). In addition, it has been shown that wild type TAP1-TAP2 complexes bind to α-[32P]-labeled 8-azido-ATP and to ATP and ADP-Sepharose beads (14, 17). Here we show that detergent extracts of isolated TAP subunits are also capable of binding nucleotide. We generated mutants of TAP1 and TAP2 that were altered at structurally analogous residues in the Walker A motif of TAP1. We observed that the TAP1(K544M) mutation significantly reduced nucleotide binding by TAP1 but that the TAP2(K509M) mutation did not significantly alter nucleotide binding by TAP2. These observations point to structural differences in the nucleotide binding pockets of TAP1 and TAP2. Indeed, the NBD of TAP1 and TAP2 show only about 51% sequence identity; thus, structural differences might be expected at the atomic level. These differences are likely to contribute to differences in the respective nucleotide binding pockets, which might further lead to differing affinities for nucleotide and differences in catalytic properties for nucleotide hydrolysis. It remains to be addressed whether such differences exist and, if so, whether there are any functional consequences.

Here we report that the K509M mutation in TAP2 abrogates peptide transport by TAP1-TAP2(K509M) complexes, although ATP binding by this mutant is not significantly different from wild type. Impairment in peptide translocation does not arise from structural disruptions induced by the mutation, since TAP1-TAP2(K509M) complexes are capable of binding both peptides and nucleotides (Figs. 1 and 3). Furthermore, based upon limited proteolytic digestion analysis, the proteolysis profiles observed for TAP1-TAP2(K509M) complexes closely parallel the profiles seen for TAP1-TAP2 complexes.2 Thus, nucleotide hydrolysis by TAP complexes containing mutant TAP2 appears to be impaired. Likewise, the TAP1(K544M) mutation reduces peptide translocation efficiency of TAP complexes even

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2 S. Arora and M. Raghavan, unpublished observations.
Table I
Comparisons of peptide binding to wild type TAP1-TAP2 or TAP1(K544M)-His-TAP2 complexes in the presence of ADP and apyrase

|             | Apparent $K_D$ (apyrase) | Apparent $K_D$ (ADP) | $k_d$ (apyrase) | $k_d$ (ADP) |
|-------------|--------------------------|----------------------|----------------|-------------|
| TAP1-TAP2   | 18.4 ± 4.8               | 28.4 ± 16            | 0.013 ± 0.005  | 0.014 ± 0.008|
| TAP1(K544M)-His-TAP2 | 17.4 ± 3.5               | 20.4 ± 12            | 0.014 ± 0.008  | 0.016 ± 0.02  |

when no residue modifications are introduced into the TAP2 subunit. Thus, nucleotide hydrolysis by TAP complexes containing mutant TAP1 also appears to be impaired. We are presently unable to directly demonstrate impaired ATPase activity of TAP complexes containing either mutant TAP subunit, since we do not have a working assay to measure the ATPase activities of TAP1 or TAP2 NBD or of TAP1-TAP2 complexes. ATPase activities have been previously demonstrated for purified NBD of other ABC transporters (for example, Refs. 31–33). Using purified TAP1 and TAP2 NBD and TAP NBD complexes (15), we were unable to unambiguously demonstrate ATPase activities. The lack of measurable ATPase activity for TAP NBD suggested that intact membrane-associated TAP complexes might be required for observation of the enzymatic activity. Expression of the human MDR1 gene in S99 cells has previously been shown to generate a membrane ATPase activity that is significantly stimulated by drugs known to interact with P-glycoprotein (34). We are attempting to develop an analogous assay using microsomal membrane preparations expressing wild type TAP1-TAP2 complexes but thus far have been unable to demonstrate a TAP-specific ATPase activity.

The observation that the TAP2(K509M) mutation impairs translocation by TAP1-TAP2(K509M) complexes although no residue alterations were introduced into TAP1 indicates that the ATPase activity at TAP1, if present, is insufficient for completion of a peptide translocation cycle. Taken together with the observation that the TAP1(K544M) mutation significantly reduces peptide translocation efficiency of TAP complexes when no residue modifications are introduced into TAP2, these results indicate a coupling between nucleotide interactions with TAP1 and TAP2. The mechanistic model that emerges from these studies differs from that recently described for histidine permease. Functional analyses of mutants of histidine permease, which contains two copies of a single NBD (HisP), have shown that presence of one intact ATP hydrolysis site is sufficient to support ATPase activity and substrate translocation (35). In chimeric histidine permease complexes containing one ATPase-active and one ATPase-inactive NBD, ligand translocation occurred at half the rate of the wild type. These observations are consistent with a mechanism for histidine permease, whereby, in wild type complexes, two molecules of ATP are hydrolyzed within a single turnover of the catalytic cycle, and hydrolysis at either NBD can result in ligand translocation. By contrast to histidine permease, in the case of the P-glycoprotein-based drug transport system, mutations of the Walker A lysine at either one NBD completely blocked drug-stimulated ATPase activity of P-glycoprotein and drug transport, although no residue alterations were introduced at the second site (19, 21). Other mutants, as well as single site chemical modifications of P-glycoprotein were observed to show similar effects (36–38). Similar results were obtained for maltose permease (39), another bacterial transporter, which contains two copies of a single NBD (MalK). When the Walker A lysine was replaced with asparagine in both MalK subunits, maltose transport and ATPase activities were reduced to 1% of those of the wild type. When the mutation was present in only one of the two subunits, the complex had 6% of the wild-type activities. Functional coupling of ATPase activities observed for P-glycoprotein and MalK might arise if ATP hydrolysis is required at two distinct steps during a single turnover of the catalytic cycle as has been recently suggested for P-glycoprotein (40, 41). Activation of the ATPase activity at each NBD might require transient conformational states that are acquired at specific steps of a translocation cycle. Modification at either ATPase site would then be expected to cause conformational trapping, resulting in an inability to complete a translocation cycle. Other models invoking alternating catalytic cycles (42) at each NBD are also consistent with the observations of functional coupling between the two NBD.

Our studies indicate that TAP may belong to a group of transporters with mechanistic similarities to P-glycoprotein and MalK. It is interesting that the TAP2(K509M) mutation abrogates peptide translocation by TAP1-TAP2(K509M) complexes but that the TAP1 mutant with a significant impairment in TAP1 nucleotide binding appears to, with low efficiency, mediate peptide translocation by TAP1(K544M)-His-TAP2 complexes. One interpretation of these observations is that nucleotide hydrolysis by TAP2 constitutes the first ATP hydrolysis step during a single peptide translocation cycle. This first hydrolytic event might be accompanied by the release of bound peptide and its translocation. Nucleotide hydrolysis by TAP1 might then be required to reset the transporter for another cycle of peptide translocation. Additional experiments are required to obtain further evidence in support of such a mechanism and to obtain greater insights into the precise role of TAP1 and TAP2 during translocation.

We analyzed the ability of each mutant TAP subunit in combination with the wild type partner to bind peptides. Using fluorescence quenching assays, the equilibrium binding constant we calculate for peptide interaction with wild type TAP1-TAP2 complexes ($K_D = 19.4 \pm 4.8 \text{ nm}$) is quite similar to that previously reported ($K_D = 12 \pm 1 \text{ nm}$) (27). However, the calculated dissociation rate constant ($k_d = 0.013 \pm 0.005 \text{ s}^{-1}$) is severalfold faster than that previously reported ($k_d = 0.002 \text{ s}^{-1}$) (27). The difference in the dissociation rate might arise because the binding experiments were carried out at room temperature and 10 °C, respectively. Using similar sets of fluorescence quenching assays, we show here that TAP1(K544M)-His-TAP2 and TAP1-TAP2(K509M) complexes are capable of binding peptides, although the binding affinity of TAP1-TAP2(K509M) complexes appears weaker than wild type.
The finding that the K544M mutation in TAP1 significantly reduces nucleotide binding also allowed us to explore the linkage between nucleotide binding by TAP1 and peptide binding to TAP1-TAP2 complexes. Using assays with fluoresecently labeled peptides, we studied peptide binding to wild type TAP1-TAP2 complexes or TAP1(K544M)-His-TAP2 complexes in the presence of apyrase (nucleotide-depleting conditions) or in the presence of ADP. For the latter experiments, microsomes were first exposed to 1 mM ADP and subsequently diluted 10-fold into buffers containing fluorescent peptide. Under these conditions, wild type TAP1 is expected to be nucleotide-bound in TAP1-TAP2 complexes, based upon previous studies of inhibition of 8-azido-ATP labeling of TAP1 in the presence of 100 μM to 1 mM cold ADP (14). For TAP1(K544M)-His-TAP2 complexes that were treated with apyrase, the TAP1(K544M)-His is expected to be significantly nucleotide-depleted, based upon the result that nucleotide binding to the TAP1(K544M)-His mutant is significantly impaired, and that apyrase might further facilitate nucleotide depletion. Since the apparent Kd and koff values for wild type TAP1-TAP2 and mutant TAP1(K544M)-His-TAP2 complexes in the presence of ADP and apyrase, respectively, are very similar, there does not appear to be a strong correlation between nucleotide binding to TAP1 and peptide binding. Our interpretation of this result is that a nucleotide-depleted TAP1 is functional for peptide binding. For the peptide binding experiments described here, we were careful to maintain the mutant TAP proteins at levels equal to or greater than that present for wild type TAP1-TAP2 complexes, at which unambiguous fluorescence quenching and recovery signals were obtained. At low expression levels of either TAP1 or TAP2, we found that a peptide binding signal could not be unambiguously discerned even for wild type TAP1-TAP2 complexes. These observations raise the question of whether expression levels of TAP1 or TAP2 in other systems could influence the results of binding experiments, which indicated that nucleotide binding is required for peptide interactions with the TAP complex (17).

It has previously been reported that the presence of ATP and nonhydrolyzable ATP analogs enhances the dissociation of peptides from TAP1-TAP2 complexes (25). At room temperature, we find no significant differences in the calculated dissociation rates for peptide-TAP1-TAP2 complexes in the presence of ADP and apyrase (Table 1). We also analyzed peptide binding to wild type TAP1-TAP2 complexes in the presence of ATP and observed that the ATP does not alter the Kd or koff values, relative to those observed in the presence of ADP and apyrase (data not shown). This result was somewhat surprising, since ATP induces peptide translocation, which entails a prior release of TAP-associated peptides. In this context, it is noteworthy that the binding experiments were carried out at room temperature, whereas the translocation experiments require a 37 °C incubation. It is possible that ATP hydrolysis is significantly inhibited at the lower temperature. The nucleotide insensitivity of peptide interactions with TAP1-TAP2 complexes at room temperature points to a mechanism in which ATP hydrolysis rather than binding per se might be associated with the structural changes that accompany the cycles of peptide binding and release. Further experiments are required to establish the occurrence of ATP-induced structural changes in TAP complexes at 37 °C and their effect on peptide binding.

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