Nucleotide Interactions with Membrane-bound Transporter Associated with Antigen Processing Proteins*

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The transporter associated with antigen processing (TAP) complex is an integral part of the major histocompatibility complex class I antigen presentation pathway (1). TAP is complex is an integral part of the major histocompatibility complex class I antigen presentation pathway (1). TAP is com-

'TAP contains two nucleotide-binding domains (NBD) in the TAP1 and TAP2 subunits. When expressed as individual subunits or domains, TAP1 and TAP2 NBD differ markedly in their nucleotide binding properties. We investigate whether the two nucleotide-binding sites of TAP1/TAP2 complexes also differ in their nucleotide binding properties. To facilitate electrophoretic separation of the subunits when in complex, we used TAP complexes in which one of the subunits was expressed as a fluorescent protein fusion construct. In binding experiments at 4 °C using the photo-cross-linkable nucleotide analogs 8-azido-[γ-32P]ATP and 8-azido-[α-32P]ADP, TAP2 was found to have reduced affinity for nucleotides compared with TAP1, when the two proteins were separately expressed. Complex formation with TAP1 enhanced the binding affinity of the TAP2 nucleotide-binding site for both nucleotides. Binding analyses with mutant TAP complexes that are deficient in nucleotide binding at one or both sites provided evidence for the existence of two ATP-binding sites with relatively similar affinities in TAP1/TAP2 complexes. TAP1/TAP2 NBD interactions appear to contribute at least in part to enhanced nucleotide binding at the TAP2 site upon TAP1/TAP2 complex formation. Binding analyses with mutant TAP complexes also demonstrate that the extent of TAP1 labeling is dependent upon the presence of a functional TAP2 nucleotide-binding site.

The transporter associated with antigen processing (TAP) complex is an integral part of the major histocompatibility complex class I antigen presentation pathway (1). TAP is comprised of two related subunits, TAP1 and TAP2, which are necessary and sufficient for peptide translocation from the cytosol into the endoplasmic reticulum (2, 3). Both proteins contain an N-terminal membrane-spanning region (MSR) and a C-terminal nucleotide-binding domain (NBD). Peptide translocation by TAP complexes is preceded by peptide binding to the cytosolic face of TAP1/TAP2 complexes, a step that appears to be nucleotide binding-independent, at least at low temperatures (4, 5). However, the presence of ATP or ADP is critical for maintaining the structural stability of TAP complexes at physiological temperatures (6), and TAP mutants that are defective in nucleotide binding at the TAP2 site lose their ability to bind peptide at 37 °C but not at lower temperatures (7). ATP hydrolysis is critical for peptide translocation across the endo
dplasmic reticulum membrane (8), and a peptide-stimulated ATPase activity has been described for TAP complexes (9).

The role of each NBD during peptide translocation is not well understood. Mutations in both NBD have been shown to affect peptide translocation efficiency, although mutations on TAP2 NBD generally have more severe consequences. Based upon mutagenesis studies at structurally analogous residues on TAP1 and TAP2, experiments from several labs have resulted in the postulation of functional distinctions between a TAP1 and a TAP2 NBD during transport (5, 7, 10, 11). Our subsequent studies indicated that chimeric TAP complexes containing two TAP1 NBD and two TAP2 NBD are capable of peptide translocation but with reduced efficiency relative to wild type complexes (12). TAP complexes containing two TAP2 NBD were less efficient in nucleotide binding and peptide translocation, relative to TAP complexes containing two TAP1 NBD. Likewise the isolated TAP2 subunit and TAP2 NBD bound nucleotides with reduced efficiency compared with the isolated TAP1 subunit and TAP1 NBD (5, 7, 11, 13). These observations raised the question of whether the TAP1 and TAP2 nucleotide-binding sites of TAP1/TAP2 complexes also had distinct nucleotide binding properties.

TAP is a member of a family of transporter proteins called ATP-binding cassette (ABC) transporters. ABC transporters have similar domain organizations and have considerable sequence identity in their NBD. Conserved sequence motifs within the NBD include the Walker A and Walker B motifs, the consensus C (signature) motif, and the switch region (reviewed in Ref. 14). There is increasing evidence in the literature that the NBD of ABC transporters interact in a manner similar to that observed in the DNA repair enzyme Rad50 (15). Indeed, recent structures of the intact ABC transporter (BtuCD) (16) and of NBD dimers of the ABC transporter MJ0796 (17) and biochemical studies analyzing the products of vanadate-cata
lyzed photo-cleavage of the maltose transporter NBD (18) have provided strong evidence in favor of a Rad50-like NBD-NBD interface for ABC transporters. In such an interaction, each ATP-binding site is comprised of residues that derive from the interface of two NBD. If TAP1 and TAP2 NBD interact in a
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Rad50-like manner, the TAP1 nucleotide-binding site would contain residues primarily from the Walker A motif of TAP1 and from the consensus C motif of TAP2, whereas the TAP2 nucleotide-binding site would include residues primarily from the Walker A motif of TAP2 and the TAP1 consensus C motif (see “Discussion”). A Rad50-like interface for ABC transporter NBD could explain the high degree of conservation of the consensus C motif. Furthermore, a Rad50-like interface provides a greater number of protein-nucleotide contacts than those observed in structures of ABC transporter NBD monomers (19–21). Therefore, it was conceivable that the nucleotide binding properties of isolated TAP1 and TAP2 subunits were not reflective of their nucleotide binding properties in complex.

Human TAP1 and TAP2 are not well resolved on SDS-PAGE gels; however, by creating a fusion protein of TAP1 and TAP2 with enhanced green fluorescent protein (TAP1-eGFP) and enhanced yellow fluorescent protein (TAP2-eYFP), respectively, the complexes of one tagged subunit and the partner untagged subunit could be easily resolved and quantified on a gel. By cross-linking radiolabeled 8-azido nucleotides at varying concentrations, we were able to derive affinity constants for 8-azido nucleotide binding to the individual TAP subunits when expressed alone and in complex. We found that in a TAP1/TAP2 complex, but not in the individual subunits, the nucleotide binding affinities derived from labeling of the TAP1 and TAP2 subunits, respectively, were almost identical. The TAP2 subunit when expressed alone had reduced affinity for both nucleotide triphosphates and nucleotide diphosphates, compared with TAP1.

EXPERIMENTAL PROCEDURES

Baculoviruses for Expression of TAP1, TAP2, TAP1/K544M, TAP2/K509M, T2MT1C, T1MT2C, TAP1-eGFP, and TAP2-eYFP—Baculoviruses encoding wild type human TAP1 and TAP2 were obtained from the laboratory of Dr. Robert Tampé (22). We have previously described the construction of baculoviruses encoding the TAP1 mutant (TAP1/K544M) and the TAP2 mutant (TAP2/K509M) (5). Construction of the chimeric TAP proteins T1MT2C (containing the TAP1 MSR and TAP2 NBD) and T2MT1C (containing the TAP2 MSR and TAP1 NBD) has also previously been described (12).

For construction of TAP1 and TAP2 fluorescent protein fusions, human TAP1 and TAP2 cDNA were obtained from Dr. John Trowsdale. Bridge PCR was used to generate the TAP1-eGFP fusion, for which the eGFP template was obtained from the pEYFP plasmid (Clontech). The first PCR amplified the TAPI portion of the fusion construct using a 5′ primer with a BglII site followed by a sequence complementary to the 5′ end of TAP1 and a 3′ primer with the last 15 nucleotides of the TAP1 N-terminal (see Methods). This PCR product was gel-extracted and used as templates for a third PCR, which used 3′ primers that had been designed to contain the TAP2 eYFP (Stratagene) and sequenced. The TAP1-eGFP fusion was then ligated into the BglII site of pAcUW51 (BD Pharmingen), which was used to generate a TAP1-eGFP-encoding baculovirus using the BaculoGold Transfection kit (BD Pharmingen). For construction of the TAP2-eYFP fusion, TAP2 in pCR2.1 (5) was first extracted by PCR using 5′ and 3′ primers that had BglII sites. Two extra nucleotides were added between the last codon of TAP2 and the 3′ BglII site to maintain the reading frame for YFP. This PCR product was gel-purified and ligated into the vector pCR Script (Stratagene). The TAP2 was then excised from pCRScript with BglII and ligated into the vector pVL1393-YP (BD Pharmingen) at the BamHI site to generate the TAP2 fusion with YFP. This vector was then used to generate a TAP2-eYFP-encoding baculovirus.

Insect Cell Culture, Microsome Preparations, and Analyses of TAP Expression—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 desired baculovirus combinations at appropriate multiplicities of infection values (usually 5–30, depending on the individual baculovirus) and incubated at 27°C for 60 h. Following these infections, microsomal membrane fractions were generated as described (22). TAP expression in the microsomes was verified by immunoblotting analyses with the TAP1-specific antibody 148.3 (22) and the TAP2-specific antibody (435.3) (23). Where comparison of the relative expression levels of the different TAP constructs was required, we used the IP3 antiseraum (24), which recognizes all of the TAP constructs described here.

Peptide Translocation Experiments—Incorporated peptide translocation experiments were performed using the desired microsome preparations as described previously (5). This procedure was based upon well characterized assays (8, 22).

8-Azido-[32P]Nucleotide Binding Experiments—Insect cell microsomes were washed three times in assay buffer (40 mM Tris, 100 mM NaCl, and MgCl2, pH 7.6) and resuspended to a final volume in the same buffer. When 8-azido-[γ-32P]ATP was used, the following ATPase inhibitors were added to the assay buffer: 5 mM NaN3, 2 mM EGTA, and 1 mM ouabain. 8-Azido-[α-32P]ATP, 8-azido-[γ-32P]ATP, or 8-azido-[α-32P]ADP (Affinity Labeling Technologies) and assay buffer were combined in a total volume of 10 μl, following which 10 μl of the washed microsomes were added. After a 15-min incubation on ice, the samples were transferred to wells of a 96-well plate on ice, cross-linked immediately with a 254-nm UV lamp for 3 min, and then transferred back into tubes. The samples were then washed three times with assay buffer, resuspended in SDS-PAGE sample buffer, and heated to 95°C for 5 min. The proteins were separated on 10% polyacrylamide gels and dried using a Bio-Rad model 483 gel drier. The dried gels were exposed to a PhosphorImager plate overnight, and labeled TAP proteins were visualized using a PhosphorImager SI (Molecular Dynamics). Quantifications were obtained using ImageQuant software. After background subtraction, the amounts of radioactivity observed were plotted and analyzed using the Prism software package (graph Pad software). The data were fitted to a one-site binding equation, \( Y = B_{max} \times X(K_p + X) \), where \( B_{max} \) is the maximal binding, \( X \) is the concentration of 8-azido nucleotide, and \( K_p \) is the binding constant for 8-azido-[32P]nucleotide. Typically, between 60 and 80% of the input radioactivity was associated with the supernatants from the first centrifugation step after cross-linking, with a higher percentage being associated at the higher concentrations of 8-azido nucleotides. Analogous binding experiments with crude membranes have previously been used to estimate the 8-azido nucleotide binding affinities of the NBD of P-glycoprotein and CFTR, respectively (25, 26).

For the ATP/ADP competition experiments, the same protocol as above was used, except that varying amounts (0.25–600 μM) of unlabeled ATP or ADP were added to the tubes before adding the microsomes. The ECPm values were derived by plotting labeling intensities corresponding to TAP1 and TAP2 as a function of unlabeled ATP and ADP concentrations.

RESULTS

Expression, Translocation Function, and 8-Azido Nucleotide Binding by Fluorescent Protein-tagged TAP Subunits or TAP1/TAP2 Complexes—We created TAP1-eGFP and TAP2-eYFP fusion constructs and expressed the proteins in insect cells using a baculovirus-based system. By expressing tagged TAP1 protein (TAP1-eGFP), tagged TAP2 protein (TAP2-eYFP), wild type TAP1, or wild type TAP2 in any combination of a TAP1 and a TAP2, we were able to generate complexes that were functional for peptide translocation. This was tested by using insect cell microsomes expressing both tagged proteins (TAP1-eGFP/TAP2-eYFP) or the individual subunits expressed singly in a radiolabeled peptide-based translocation assay. Indeed, the tagged TAP complex was able to translocate peptides, whereas the individual subunits were not (Fig. 1B). TAP complexes expressing one tagged subunit and one wild type subunit (TAP1-eGFP/TAP2 and TAP1/TAP2-eYFP) were also competent for peptide translocation (data not shown).

We next examined 8-azido-[α-32P]nucleotide binding to individual TAP subunits or TAP complexes in which one of the subunits was expressed as a fluorescent protein fusion construct. To examine 8-azido-[α-32P]nucleotide binding in the absence of detergent, we incubated insect cell microsomes expressing one or both TAP subunits or neither protein with 8-azido-[α-32P]ATP for 15 min at 4°C, followed by UV-induced covalent cross-linking of bound 8-azido-[α-32P]nucleotides. The
membranes were washed to remove unbound nucleotide. The proteins were separated by SDS-PAGE, and proteins that had bound 8-azido-[α-32P]nucleotides were visualized using phosphorimaging analyses of dried gels. Compared with microsomes derived from uninfected cells, additional labeled bands were visualized in microsomes expressing one or both TAP subunits at the expected molecular weights corresponding to the wild type subunits and/or that corresponding to the fluorescent protein fusion constructs (Fig. 1D, lane 1 compared with other lanes). The use of the fluorescent protein epitope tags allowed for adequate separation of the TAP subunits by SDS-PAGE and for visualization of the labeling of individual components of the TAP1/TAP2 complex by 8-azido-[α-32P]nucleotides (Fig. 1D, lane 2). Labeling of TAP subunits was inhibitable by unlabeled ATP and ADP, with derived EC50 values in the low micromolar range (Fig. 1, E and F, respectively).

8-Azido Nucleotide Binding to TAP Subunits Expressed Individually and in Combination—For all of the ATP binding analyses described here on in the manuscript, we used 8-azido-[γ-32P]ATP rather than 8-azido-[α-32P]ATP, because we found that insect cell microsomal membrane preparations had measurable ATPase activity, even at 4°C in the presence of the ATPase inhibitors ouabain, EGTA, and sodium azide (regardless of TAP expression). Although 8-azido-[γ-32P]ATP would be expected to be hydrolyzed as efficiently as 8-azido-[α-32P]ATP, labeling by 8-azido-[γ-32P]ATP would report just on nucleotide triphosphate binding to TAP complexes. Microsomal membrane fractions were prepared of insect cells infected with viruses encoding single TAP subunits (Fig. 2A), or TAP1/TAP2 combinations (Fig. 2B and C). For examining nucleotide binding by TAP2 when in complex, the TAP1 component was expressed in excess (Fig. 2B), and for examining nucleotide binding by TAP1 when in complex, the TAP2 component was expressed in excess (Fig. 2C).

The membranes were incubated with different concentrations of photo-cross-linkable nucleotide analog 8-azido-[γ-32P]ATP, followed by UV cross-linking and analyses of TAP labeling by SDS-PAGE and phosphorimaging analyses (Fig. 2, D–F). The extent of labeling of the TAP subunits was quantified using ImageQuant software, plotted as a function of the input 8-azido-[γ-32P]ATP concentration, and then fitted to a one-site binding model to derive KD values corresponding to TAP1 and TAP2 labeling, when expressed individually or in combination (Fig. 2, G–I).

TAP2-eYFP (or TAP2; data not shown), when expressed separately, had reduced 8-azido-ATP binding affinity (KD = 19.3 ± 2.5 μM) compared with TAP1 (KD = 4.6 ± 1.9 μM) (data not shown) expressed alone (Fig. 2G and Table I). When TAP2 is complexed with excess TAP1-eGFP, the affinity corresponding to the TAP2 labeling is increased (KD = 2.7 ± 1 μM) (Fig. 2H and Table I). Mutation of the TAP2 Walker A lysine residue (TAP2(K509M)) reduced the TAP2-associated signal and derived affinity (KD > 20 μM) when expressed in complex with TAP1-eGFP (Fig. 2, E, bottom panel compared with top panel, and H and Table I). Thus, the 8-azido-ATP binding affinity derived for TAP2 in the wild type TAP1-eGFP/TAP2 complexes (KD = 2.7 ± 1 μM) must correspond to binding
via residues in the TAP2 Walker A motif. These studies indicated that the nucleotide binding affinity of the isolated TAP2 subunit was lower than that of the TAP1 counterpart but that TAP1/TAP2 complex formation induced changes that enhanced the nucleotide binding affinity at the TAP2 site.

When TAP1-eGFP is co-expressed with excess TAP2 (Fig. 2, C, F, and I), the affinity corresponding to TAP1-eGFP labeling was 2.1 ± 0.8 μM (Fig. 2I and Table I). In the corresponding TAP1-eGFP/TAP2/K509M complexes, a similar affinity was derived corresponding to TAP1-eGFP labeling ($K_D = 2.8 \pm 0.1$ μM) (Fig. 2I and Table I). Thus, high affinity binding by TAP1-eGFP is visualized even in the absence of high affinity nucleotide binding to TAP2. However, the signals derived for TAP1-eGFP in the TAP1-eGFP/TAP2(K509M) mutant complex are reduced compared with that derived for the wild type complex (Fig. 2I), even though slightly higher levels of TAP1-eGFP were present in the mutant complex (Fig. 2C, lane 1 compared with lane 2). Taken together, the observations described so far indicate that both nucleotide-binding sites of TAP1/TAP2 complexes have similar affinities for 8-azido nucleotides.

### Table I

| Nucleotide Binding by the TAP1/TAP2 Complex | $K_D$(ATP) | $K_D$(ADP) |
|------------------------------------------|--------|--------|
| TAP2 nucleotide binding                  |        |        |
| TAP2-eYFP                                | 19.3 ± 2.5 | 4.4 ± 1.4 |
| TAP2/TAP1-eGFP                           | 2.7 ± 1.0 | 0.5 ± 0.1 |
| TAP2/K509M/TAP1-eGFP                     | >20 | 9.0 ± 1.4 |
| TAP1 nucleotide binding                   |        |        |
| TAP1                                     | 4.6 ± 1.9 | 1.4 ± 0.1 |
| TAP1-eGFP/TAP2                           | 2.1 ± 0.8 | 0.7 ± 0.1 |
| TAP1-eGFP/TAP2(K509M)                    | 2.8 ± 2.9 | 0.6 ± 0.2 |

Fig. 2. Complex formation with TAP1 markedly increases the 8-azido-ATP binding affinity of the TAP2 nucleotide-binding site. Insect cell microsomal membranes expressing TAP1 alone or TAP2-eYFP alone (A), the TAP1-eGFP/TAP2 or TAP1-eGFP/TAP2(K509M) combinations with the TAP1-eGFP component in excess (B), or the TAP1-eGFP/TAP2 or TAP1-eGFP/TAP2(K509M) combinations with the TAP2 or TAP2(K509M) components in excess (C) were incubated with different concentration of 8-azido-[γ-32P]ATP for 15 min on ice and subsequently cross-linked by UV irradiation. The proteins were then separated by 10% SDS-PAGE, and radiolabeling of TAP subunits was visualized by phosphorimaging analyses. A–C, immunoblotting analyses to visualize one or both TAP proteins. The IP3 antibody recognized both TAP1 and TAP2. D–F, phosphorimaging analyses of labeled TAP proteins. G–I, quantification of labeled TAP bands and fitting to a one site binding model. The $K_D$ values corresponding to labeling of the indicated TAP subunit(s) were derived from multiple independent analyses, summarized in Table I.
Fig. 3. 8-Azido-ATP binding affinities at the two nucleotide-binding sites of TAP1/TAP2-eYFP complexes or chimeric TAP complexes containing two TAP1 NBD or two TAP2 NBD. Insect cell microsomal membranes expressing TAP1/TAP2-eYFP, T1MT2C/TAP2-eYFP (two TAP2 NBDs), or TAP1-eGFP/T2MT1C (two TAP1 NBDs) were incubated with different concentration of 8-azido-[γ-32P]ATP for 15 min on ice and subsequently cross-linked by UV irradiation. The proteins were then separated by 10% SDS-PAGE, and radioactive labeling of TAP subunits was visualized by phosphorimaging analyses. A, immunoblotting analyses to visualize components of each complex. B, phosphorimaging analyses of labeled TAP proteins. C, quantification of labeled TAP bands and fitting to a one-site binding model. The indicated $K_v$ values are the averages of two or three independent sets of analyses.

The data shown in Fig. 2 were derived from analyses of 8-azido-[γ-32P]ATP binding to TAP1/TAP2 complexes. A similar set of analyses was also carried out using 8-azido-[γ-32P]ADP, and analogous results were obtained (Table I). TAP2-eYFP had reduced affinity for 8-azido-[γ-32P]ADP compared with TAP1 when the two subunits were separately expressed. Complex formation with TAP1 increased the binding affinity corresponding to TAP2 labeling by 9-fold (Table I), whereas complex formation with TAP2 increased the binding affinity corresponding to TAP1 labeling by only 2-fold (Table I).

**TAP1/TAP2 NBD Interaction Appear to Contribute at Least in Part to Enhanced Nucleotide Binding at the TAP2 Site upon TAP1/TAP2 Complex Formation**—The marked affinity enhancement at the TAP2 nucleotide-binding site upon TAP1/TAP2 complex formation could result from a general stabilization of the TAP2 structure upon TAP1/TAP2 complex formation or, more specifically, result from NBD-NBD interactions that allow a greater set of nucleotide contacts at the TAP2 site of TAP1/TAP2 complexes compared with free TAP2. To distinguish these possibilities, we compared affinities corresponding to TAP1 and TAP2 labeling in TAP1/TAP2-eYFP complexes as well as T1MT2C/TAP2-eYFP complexes. T1MT2C is a chimeric protein containing the MSR of TAP1 and the NBD of TAP2 (12). We have previously shown that this chimera forms complexes with TAP2 and that these complexes are functional for peptide translocation, although with reduced efficiency relative to wild type. The affinity corresponding to TAP2-eYFP labeling was $1.8 \pm 1.4 \mu M$ in TAP1/TAP2-eYFP complexes, compared with $11.4 \pm 2.4 \mu M$ in T1MT2C/TAP2-eYFP complexes (6-fold different; Fig. 3C, first and second panels). As expected, the affinity corresponding to T1MT2C labeling was also reduced in T1MT2C/TAP2-eYFP complexes ($25 \pm 14.2 \mu M$) (Fig. 3). These observations suggested that TAP1/TAP2 NBD/NBD interactions are important for optimal nucleotide binding at the TAP2 nucleotide-binding site and that TAP2/TAP2 NBD interactions or TAP1/TAP2 MSR interactions are not fully sufficient for affinity enhancement at the TAP2 site.

We also examined 8-azido-ATP binding by TAP1-eGFP/T2MT1C complexes (Fig. 3, B and C, third panel). T2MT1C is a chimeric construct containing the MSR of TAP2 and the NBD of TAP1 (12). We previously showed strong binding of TAP1/T2MT1C complexes to ATP and ADP agarose beads compared with TAP2/T1MT2C complexes, which correlated with enhanced translocation efficiency of the TAP1/T2MT1C complexes. Measurement of 8-azido-ATP binding to TAP1-eGFP/T2MT1C complexes indicated an affinity of $0.5 \pm 0.3 \mu M$ corresponding to T2MT1C labeling and $0.4 \pm 0.1 \mu M$ corresponding to TAP1-eGFP labeling. Interestingly, at comparable expression levels, the intensity of labeling of the T2MT1C construct was significantly higher than that of TAP1-eGFP (Fig. 3, B and C, third panel), a reversal of the trend observed with TAP1-eGFP/TAP2 complexes (Figs. 2F and 3, B and C, top panels). Furthermore unlike TAP1, T2MT1C bound 8-azido-ATP with high affinity regardless of being complexed with TAP1 (data not shown). Thus, whereas high affinity nucleotide binding to a TAP1 NBD is observed both in the presence and absence of a TAP2 NBD, high affinity nucleotide binding to a TAP2 NBD requires the presence of a TAP1 NBD.

**Labeling of Nucleotide Binding-deficient TAP1 Is Dependent upon the Presence of a Functional TAP2 Nucleotide-binding Site**—The above observations raised the possibility that the presence of TAP1 residues at the TAP2 nucleotide-binding site could be responsible for the observed affinity enhancement at the TAP2 site upon TAP1/TAP2 complex formation. To further explore this possibility and also investigate the effect of TAP1 nucleotide binding upon TAP1 labeling, we examined 8-azido-ATP binding by a mutant TAP complex containing a defective TAP1 nucleotide-binding site (Fig. 4). We previously showed that mutation of the Walker A lysine of TAP1 (TAP1(K544M)) resulted in significantly reduced binding of single subunit TAP1 to ATP- and ADP-agarose beads (5). Surprisingly, however, when TAP1(K544M) was expressed in combination with TAP2-eYFP, the labeling intensities observed for TAP1(K544M) and TAP2-eYFP were almost identical over the entire concentration range, and thus, nearly identical affinities were derived ($3.8 \pm 3.5$ and $4.0 \pm 2.5 \mu M$, corresponding to TAP1(K544M) and TAP2-eYFP labeling, respectively; Fig. 4, A–C). The parent TAP1/TAP2-eYFP complexes yielded $K_v$ values of $1.5 \pm 0.4$ and $1.3 \pm 0.5 \mu M$, corresponding to labeling of TAP1 and TAP2-eYFP, respectively (data not shown).

We also investigated labeling of TAP1(K544M) in the absence of a functional TAP2 nucleotide-binding site. For these analyses, we prepared microsomes containing TAP1(K544M)/TAP2 complexes and TAP1(K544M)/TAP2(K509M) complexes. Because only untagged versions (but not fluorescent protein fusions) of the mutant TAP subunits were available, only the combined signal on both the TAP1 and TAP2 components could be visualized with these complexes (Fig. 4, D and E). At comparable expression levels of both components (Fig. 4D), strong labeling was visualized for the TAP1(K544M)/TAP2 combination, whereas signals for the TAP1(K544M)/TAP2(K509M) combination were barely detectable (Fig. 4E, top and middle panels, respectively). These results demonstrated that the TAP1(K544M) component by itself did not bind very efficiently to 8-azido-[γ-32P]ATP, consistent with our previous report that the TAP1(K544M) mutant was impaired in binding to ATP-agarose and ADP-agarose beads relative to wild type TAP1 (5). Most interestingly, although distinct labeled bands corresponding to TAP1(K544M) were observed in TAP1(K544M)/TAP2-
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Fig. 4. Labeling of nucleotide binding-deficient TAP1 is dependent on the presence of a functional TAP2 nucleotide-binding site. Labeling procedures were similar to that described in the legend to Fig. 2. A and D, immunoblotting analyses of microsomes used in binding analyses shown in B and E, respectively. B, phosphorimaging analyses of 8-azido-[γ-32P]ATP binding by the TAP1(K544M) and TAP2-eYFP components of TAP1(K544M)/TAP2-eYFP complexes. C, quantification of signals from B and estimation of \( K_{D} \) values. The indicated \( K_{D} \) values are the averages of three independent sets of analyses. The \( K_{D} \) values derived for a corresponding set of analyses with wild type TAP1/TAP2-eYFP complexes were 1.5 ± 0.4 \( \mu \)M (for TAP1) and 1.3 ± 0.5 \( \mu \)M (for TAP2-eYFP). D, phosphorimaging analyses of 8-azido-[γ-32P]ATP binding to microsomes containing TAP1(K544M)/TAP2 (top panel), TAP1(K544M)/TAP2(K509M) (middle panel), or TAP1(K544M)/TAP2-eYFP (bottom panel). Signals corresponding to TAP1(K544M) could be observed when in complex with TAP2-eYFP but not when in complex with TAP2(K509M). The absence of a signal was not due to the expression level, as TAP1(K544M) was expressed at higher levels in the microsomes with the TAP2(K509M) combination compared with the TAP2-eYFP combination (see D).

DISCUSSION

TAP1 and TAP2 NBD sequences are ~60% identical. Previous studies have shown that the two NBD interact differently with nucleotide-agarose beads, with constructs containing TAP1 NBD binding strongly, and with constructs containing TAP2 NBD interacting weakly with nucleotide-agarose beads (12, 27). In the present studies, we examined nucleotide binding to the TAP1 and TAP2 sites, when the proteins were in complex. We constructed C-terminal fusions of TAP1 and TAP2 with eGFP and eYFP, respectively. When either fusion protein was paired with a wild type partner, the resulting complexes were functional and could be electrophoretically separated from the partner wild type subunit, allowing measurements of the binding of various 8-azido-adenosine nucleotides to TAP subunits when in complex.

The presence of the eGFP or eYFP fusion constructs did not influence the derived affinity, because similar affinities were observed for TAP1 and TAP1-eGFP or for TAP2 and TAP2-eYFP, both when expressed individually or as a complex. The nucleotide binding affinity of the isolated TAP2 subunit was found to be reduced compared with the TAP1 counterpart. However, TAP1/TAP2 complex formation induced changes that significantly enhanced the affinity of the TAP2 nucleotide-binding site for both 8-azido-ATP and 8-azido-ADP. TAP1/TAP2 complex formation has a less significant effect upon the affinity of the TAP1 nucleotide-binding site for 8-azido-ATP and 8-azido-ADP (Table I).

Using TAP1-eGFP/TAP2(K509M) complexes under conditions of TAP1 excess or TAP2 excess (Fig. 2), we determined that the two nucleotide-binding sites of TAP1/TAP2 complexes did in fact bind 8-azido-ATP with apparent affinities that were, within the error of these measurements, quite similar to each other. The \( EC_{50} \) values derived from inhibition analyses with ATP (Fig. 1E) were quite similar to the \( K_{D} \) values derived from direct 8-azido-[γ-32P]ATP binding analyses (Table I). The \( EC_{50} \) values derived from inhibition analyses with ADP (Fig. 1F) were higher than the \( K_{D} \) value derived from the direct 8-azido-[γ-32P]ADP binding analyses (Table I). Thus, it is possible that 8-azido-ADP does bind to TAP complexes with higher affinity than ADP, as reported for other ABC transporters.

Functional differences between TAP1 and TAP2 have previously been attributed to differences in nucleotide binding to the TAP1 and TAP2 sites of TAP1/TAP2 complexes. Based upon studies with rat TAP1 and TAP2, it has been suggested that TAP1 binds ATP more efficiently than does TAP2, whereas the binding of ADP by the two chains is essentially equivalent (10). Our present studies demonstrate that the affinities at the TAP1 and TAP2 sites for both nucleotides are in fact quite similar when TAP1 and TAP2 are in complex (Table I). By contrast, analogous studies with CFTR have indicated that the...
two NBD bind equivalently to 8-azido-ADP but differed in their interactions with 8-azido-ATP. The binding curves for 8-azido-ATP showed the occurrence of simple saturable binding at NBD1 but a sigmoidal profile at NBD2, suggesting that the two NBDs of CFTR had distinct nucleotide binding properties (25). These observations suggest the possibility of mechanistic differences between the TAP and CFTR catalytic cycles.

The affinity enhancement at the TAP2 nucleotide-binding site upon TAP1/TAP2 complex formation could result from a general stabilization of the TAP2 structure upon TAP1/TAP2 complex formation or, more specifically, could be due to NBD-NBD interactions that alter the nucleotide interaction properties at the TAP2 site. The observations of enhanced nucleotide binding by TAP2-eYFP in TAP1/TAP2-eYFP complexes compared with T1MT2C/TAP2-eYFP complexes (Fig. 3) argue for a role for NBD-NBD interactions in determining the binding affinity of the TAP2 nucleotide-binding site. In a model of the TAP2 nucleotide-binding site based upon MJ0796 dimer structure (17), several residue differences are predicted when the opposing NBD has a TAP2 sequence rather than a TAP1 sequence (Fig. 5). Apparently, the sum of such differences accounts for the observed reduction in affinity (Fig. 3) when a TAP2-TAP1 NBD-NBD interface is altered to a TAP2-TAP2 NBD-NBD interface.

Mutation of the TAP2 Walker A lysine residue (TAP2(K509M)) indeed influenced nucleotide binding at the TAP2 site (Fig. 2). This effect of the TAP2 Walker A mutation was not apparent in previous analyses of the binding to nucleotide agarose beads of single subunit wild type and mutant TAP2 (5, 11), presumably because of the low binding affinities of both wild type and mutant TAP2. The corresponding mutation in TAP1 (TAP1(K544M)) had a marked effect upon the binding of single subunit TAP1 to nucleotide agarose beads (5, 7). Surprisingly, we found that when the TAP1 Walker A mutant TAP1(K544M) was expressed in combination with TAP2-eYFP, the signal intensities as well as the affinities corresponding to TAP1 and TAP2 labeling were nearly identical (Fig. 4). This was apparent in analyses with both 8-azido-ATP (Fig. 4) and 8-azido-ADP (data not shown). However, co-expression of TAP1(K544M) with TAP2(K509M) resulted in a nucleotide-binding deficient complex (Ref. 7 and Fig. 4). These observations demonstrate a profound influence of nucleotide binding by TAP2 upon TAP1 labeling under conformational conditions that are prevalent in TAP1(K544M)/TAP2 complexes.

What mechanisms could be responsible for enhanced TAP1 labeling in TAP1(K544M)/TAP2-eYFP complexes compared with TAP1(K544M)/TAP2(K509M) complexes? In Rad50 and MJ0796-like model of NBD-NBD interactions, ATP binding was required to promote NBD dimerization (Fig. 6A) (15, 17). If TAP2-NBD interactions resemble Rad50 and MJ0796, it is conceivable that disruption of ATP binding by Walker A mutations at the TAP1 site or TAP2 site could result in disrupted NBD-NBD contacts at the mutated nucleotide-binding site. In the case of the mutant TAP1(K544M)/TAP2 complexes, a concurrent “closed” conformation at the TAP2 nucleotide-binding site could result in labeling of TAP1(K544M) residues (in the vicinity of the consensus C motif) that are in close proximity to the TAP2 nucleotide-binding site (Fig. 6B). An alternative possibility is that nucleotide binding at the TAP2 site could enhance nucleotide binding at the TAP1 site by increasing the affinity or accessibility of the TAP1 nucleotide-binding site (Fig. 6B). When we compared 8-azido-[γ-32P]ATP binding by TAP1-eGFP in TAP1-eGFP/TAP2 complexes and TAP1-eGFP/TAP2(K509M) complexes, we found that the TAP1-eGFP labeling intensity was enhanced when in complex with wild type TAP2 compared with TAP2(K509M). Importantly, however, the binding affinity was unchanged (Fig. 2D). These results indicated that nucleotide binding at the TAP2 site could enhance nucleotide binding at the TAP1 site by inducing a more “open” (nucleotide-accessible) conformation at the TAP1 nucleotide-binding site (but not by an affinity alteration at the TAP1 site). We observed that the derived affinity corresponding to TAP1(K544M) labeling in TAP1(K544M)/TAP2-eYFP complexes was nearly identical to that corresponding to TAP2-eYFP labeling (Fig. 4C) and significantly higher than that measured in TAP1(K544M)/TAP2(K509M) (Fig. 4E, middle panel; Kp cannot be estimated because significant labeling was not visualized). Thus, the TAP2(K509M) mutation appears to have distinct effects on labeling of TAP1 compared with TAP1(K544M). A likely explanation for this apparent discrepancy is that there are two possible high affinity nucleotide-binding sites in TAP1: one in the vicinity of its Walker A sequence and a second in the vicinity of its consensus C sequence. Labeling of the latter site is dependent upon the presence of a functional TAP2 nucleotide-binding site, whereas the labeling of the first site is independent of a functional TAP2

Fig. 5. Proposed architectures of the TAP2 nucleotide-binding site of TAP1/TAP2 complexes (A) compared with T1MT2C/TAP2 complexes (two TAP2 NBD) (B), based upon the structure of the MJ0796 dimer (17). Residues predicted to be involved in nucleotide contacts from the Walker A end and the Consensus C end are indicated for each site, and the residues involved in protein-protein contacts are indicated in the extreme left of each panel. The C-8 carbon of a TAP2-bound nucleotide is predicted to be in close proximity to Tyr477 of TAP2 as well as Gln642 of TAP1. 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 explain the results with TAP1(K544M)/TAP2-eYFP complexes (Fig. 4).
site. Disruption of the first site by the K544M mutation renders high affinity TAP1(K544M) labeling dependent upon the presence a functional TAP2 site. In a model of TAP1/TAP2 based upon the MJ0796 structure (17), the C-8 carbon of a TAP2-bound nucleotide is predicted to be in close proximity to Tyr477 of TAP2 as well as Glu642 of TAP1 (Fig. 5A). 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 explain the results with TAP1(K544M)/TAP2-eYFP complexes (Fig. 4).

The above arguments raise the possibility of TAP1 residues being in close proximity to the TAP2 nucleotide-binding site of TAP1/TAP2 complexes, under some conformational conditions, such as that trapped in TAP1(K544M)/TAP2-eYFP complexes. High affinity labeling of TAP1(K544M) residues in TAP1(K544M)/TAP2-eYFP complexes (Fig. 4), but not of TAP2(K509M) residues in TAP1-eGFP/TAP2/K509M complexes (Fig. 2), might arise because of conformational differences between the two mutant complexes. Alternatively, structural differences between the TAP1 and TAP2 nucleotide-binding sites, such as differences in the consensus C residues (Fig. 6), could influence the extent of NBD-NBD interaction at each nucleotide-binding site. It remains to be defined whether other conformational conditions exist in which TAP2 residues are in close proximity to the TAP1 nucleotide-binding site. Additionally, it remains to be established whether TAP1 residues are in close proximity to the TAP2 nucleotide-binding site, in resting state wild type TAP1/TAP2 complexes. Reanalyses of the TAP1 binding data in Fig. 2F (for TAP1-eGFP/TAP2) using a two-site binding model (rather than a single site binding model) did not yield better $R^2$ values. Furthermore, consistent $K_D$ values were not derived in two-site binding analyses of three independent experimental data sets. The analysis is somewhat complicated by the result that the TAP1 and TAP2 sites have nearly identical affinities TAP1/TAP2 complexes (Fig. 2) and thus do not allow us to distinguish between two-site or single-site binding models for TAP1 in TAP1-eGFP/TAP2 complexes. Additional experiments will be required to demonstrate the exact proximity of TAP1 consensus C (and other residues) to the TAP2 nucleotide-binding site, and vice versa, under different conformational conditions.

Although the affinities derived for the TAP1 and TAP2 nucleotide-binding sites of TAP1/TAP2 complexes were very similar, we observed that the signal intensities were generally higher for TAP1 labeling compared with TAP2 labeling, even if the TAP2 expression was higher (for example Fig. 2). Many factors could influence the observed labeling intensity, including the 8-azido-ATP coupling efficiency (which could vary as a function of the exact chemical environment of a given nucleotide-binding site), and the relative populations of active proteins (stability). It is also possible, as suggested in Fig. 6B, that different conformational states of TAP exist in which the two nucleotide-binding sites vary in their nucleotide accessibilities. In the resting state of TAP1/TAP2 complexes, the TAP2 site could be in a more closed (nucleotide-inaccessible) conformation compared with the TAP1 site, which would result in lower efficiency TAP2 labeling compared with TAP1 labeling. Although our studies clearly demonstrate that the inherent binding affinities of the two sites are very similar in TAP1/TAP2 complexes, more information is required pertaining to the accessibility of each site in resting state and transition state conformations.

The analyses undertaken here also allow for a reassessment of the effects of TAP1(K544M) and TAP2(K509M) mutations upon peptide binding to TAP1/TAP2 complexes. We show here as previously suggested (7) that both mutations significantly reduce the nucleotide binding affinities at the corresponding nucleotide-binding sites. Both mutant complexes were found to bind TAP-specific peptides with high affinity at room temperature (5); however, the binding affinity of the TAP1(K544M)/TAP2 complex ($K_D = 17.4 \pm 4.8 \text{ nM}$) was very similar to wild type ($K_D = 19.4 \pm 4.8 \text{ nM}$), the affinity of the TAP1/TAP2(K509M) was $-2$-fold reduced ($K_D = 39.2 \pm 5.9 \text{ nM}$). Thus, a $-2$-fold or greater reduction in the nucleotide binding affinity at the TAP2 site has a small effect on the peptide binding affinity, whereas nucleotide binding at the TAP1 site does not appear to influence peptide binding. Other studies found that a defective TAP2 nucleotide-binding site resulted in a loss of peptide binding at 37°C (but not at low temperatures), which was interpreted as indicating that nucleotide binding to TAP2 was required for peptide binding (7). Our observation is that nucleotide binding to TAP1/TAP2 complexes significantly enhances the thermostability of the TAP peptide-binding site (28) but that nucleotide binding per se is nonessential for peptide binding, because mutant TAP complexes are capable of peptide binding with high affinity at reduced temperatures. No such stability model is sufficient to explain why mutant TAP complexes containing defective TAP2 nucleotide-binding sites are defective for peptide binding when expressed in mammalian cells that are cultured at 37°C (10, 11).

In summary, we demonstrate here (i) that TAP1 and TAP2 NBD differ markedly in their nucleotide binding properties...
when expressed as individual subunits but not when expressed as a complex, (ii) that TAP1/TAP2 NBD-NBD interactions are critical for optimal nucleotide binding to the TAP2 site of TAP1/TAP2 complexes, and (iii) that the extent of TAP1 labeling is dependent upon the presence of a functional TAP2 nucleotide-binding site. Although these studies establish that the TAP1 and TAP2 nucleotide-binding sites of TAP1/TAP2 complexes are both capable of binding nucleotides with similar affinities, we cannot presently assess whether the two sites differ in nucleotide accessibility in resting state TAP1/TAP2 complexes. Furthermore, whether both sites are capable of ATP hydrolysis also remains to be established. Our findings that chimeric TAP1/TAP2 complexes containing two TAP1 NBD were functional for peptide translocation (with reduced efficiency relative to wild type TAP1/TAP2 complexes) indicated that a TAP1 NBD is capable of ATP hydrolysis, at least when the opposing NBD has the TAP1 sequence (12). Likewise, TAP complexes containing two TAP2 NBD were also functional for peptide translocation, although with low efficiency. These studies have suggested that both nucleotide-binding sites of TAP1/TAP2 complexes can hydrolyze ATP. However, this remains to be unambiguously established using wild type TAP1/TAP2 complexes.

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REFERENCES
1. Pamer, E., and Cresswell, P. (1998) Annu. Rev. Immunol. 16, 323–358
2. Abele, R., and Tampe, R. (1999) Biochim. Biophys. Acta 1461, 405–419
3. Momburg, F., and Hammerling, G. J. (1998) Adv. Immunol. 68, 191–256
4. Andrei, M. J., Ortmann, B., van Endert, P. M., Spies, T., and Cresswell, P. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 12716–12720
5. Lapinski, P. E., Neubig, R. R., and Raghavan, M. (2001) J. Biol. Chem. 276, 7526–7533
6. van Endert, P. M. (1999) J. Biol. Chem. 274, 14632–14638
7. Saveanu, L., Daniel, S., and van Endert, P. M. (2001) J. Biol. Chem. 276, 22107–22113
8. Neefjes, J. J., Momburg, F., and Hammerling, G. J. (1993) Science 261, 769–771
9. Gorbulev, S., Abele, R., and Tampe, R. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 3732–3737
10. Alberts, P., Daumke, O., Deverson, E. V., Howard, J. C., and Knittler, M. R. (2001) Curr. Biol. 11, 242–251
11. Karttunen, J. T., Lehner, P. J., Gupta, S. S., Hewitt, E. W., and Cresswell, P. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 7431–7436
12. Allan, S., Lapinski, P. E., and Raghavan, M. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 7241–7246
13. Lapinski, P. E., Miller, G. G., Tampe, R., and Raghavan, M. (2002) J. Biol. Chem. 275, 6831–6840
14. J. Biol. Chem. 1998 273, 11130–11137
15. Hopfner, K. P., Karcher, A., Shin, D. S., Craig, L., Arthur, L. M., Carney, J. P., and Tainer, J. A. (2000) Cell 101, 789–800
16. Locher, K. P., Lee, A. T., and Rees, D. C. (2002) Science 296, 1091–1098
17. Smith, P. C., Karpowich, N., Millen, L., Moody, J. E., Rosen, J., Thomas, P. J., and Hunt, J. F. (2002) Mol. Cell 10, 139–149
18. Fetsch, E. E., and Davidson, A. L. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 9685–9690
19. Yuan, Y. R., Blecker, S., Martinskevich, O., Millen, L., Thomas, P. J., and Hunt, J. F. (2001) J. Biol. Chem. 276, 32313–32321
20. Hung, L. W., Wang, I. X., Nikaido, K., Liu, P. Q., Ames, G. F., and Kim, S. H. (1998) Nature 396, 703–707
21. Gaudet, R., and Wiley, D. C. (2001) EMBO J. 20, 4964–4972
22. Meyer, T. H., van Endert, P. M., Uebel, S., Ehring, B., and Tampe, R. (1994) FEBS Lett. 351, 445–447
23. van Endert, P. M., Tampe, R., Meyer, T. H., Tisch, R., Bach, J. P., and McDevitt, H. O. (1994) Immunity 1, 491–500
24. Nijenhuis, M., and Hammerling, G. J. (1996) J. Immunol. 157, 5467–5477
25. Aleksandrov, L., Aleksandrov, A. A., Chang, X. B., and Riordan, J. R. (2002) J. Biol. Chem. 277, 15419–15425
26. Sauna, Z. E., Smith, M. M., Muller, M., and Ambudkar, S. V. (2001) J. Biol. Chem. 276, 21199–21208
27. Daumke, O., and Knittler, M. R. (2001) Eur. J. Biochem. 268, 4776–4786
28. Raghuraman, G., Lapinski, P. E., and Raghavan, M. (2002) J. Biol. Chem. 277, 41786–41794
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