Distinct Functions of the ATP Binding Cassettes of Transporters Associated with Antigen Processing

A MUTATIONAL ANALYSIS OF WALKER A AND B SEQUENCES*

Loredana Saveanu, Soizic Daniel, and Peter M. van Endert‡

From INSERM U25, 161 rue de Sevres, 75743 Paris cedex 15, France

Received for publication, December 13, 2000, and in revised form, February 28, 2001
Published, JBC Papers in Press, April 4, 2001, DOI 10.1074/jbc.M011221200

The transporters associated with antigen processing (TAP1/TAP2) provide peptides to MHC class I molecules in the endoplasmic reticulum. Like other ATP-binding cassette proteins, TAP uses ATP hydrolysis to power transport. We have studied peptide binding to as well as translocation by TAP proteins with mutations in the Walker A and B sequences that are known to mediate ATP binding and hydrolysis. We show that a mutation in the TAP1 Walker B sequence reported to abrogate class I expression by a lung tumor does not affect ATP binding affinity, suggesting a defect restricted to ATP hydrolysis. This mutation reduces peptide transport by only 50%, suggesting that TAP function can be highly limiting for antigen presentation in non-lymphoid cells. Single substitutions in Walker A sequences (TAP1K544A, TAP2K509A), or their complete replacements, abrogate nucleotide binding to each subunit. Although all of these mutations abrogate peptide transport, they reveal distinct roles for nucleotide binding to the two transporter subunits in TAP folding and in regulation of peptide substrate affinity, respectively. Alteration of the TAP1 Walker A motif can have strong effects on TAP1 and thereby TAP complex folding. However, TAP1 Walker A mutations compatible with correct folding do not affect peptide binding. In contrast, abrogation of the TAP2 nucleotide binding capacity has little or no effect on TAP folding but eliminates peptide binding to TAP at 37 °C in the presence of nucleotides. Thus, nucleotide binding to TAP2 but not to TAP1 is a prerequisite for peptide binding to TAP. Based on these results, we propose a model in which nucleotide and peptide release from TAP are coupled and followed by ATP binding to TAP2, which induces high peptide affinity and initiates the transport cycle.

The transporters associated with antigen processing (TAP)1 belong to the family of ATP binding cassette (ABC) transporters, a large group of proteins that use energy provided by nucleotide triphosphates to translocate a vast variety of substrates across intracellular or cell surface membranes (1). All ABC transporters possess two transmembrane domains, each generally composed of six membrane-spanning segments, and two nucleotide binding domains (NBDs) with primary sequence homology across the protein family. Whereas substrate interaction is generally thought to involve the transmembrane domains, the NBDs bind and hydrolyze ATP. Both of the latter events have been shown to lead to conformational changes that upon transmission to substrate binding domains in an undefined fashion result in substrate translocation (2).

Given the important role of ABC transporters in diseases such as mucoviscidosis or cancer, the mechanism of substrate transport has been subject to intense scrutiny. Mutagenesis studies and crystallographic analysis of HisP and MalK, the NBDs of bacterial histidine and maltose transporters, respectively, have elucidated the role of several conserved sequence motifs contained in ABC transporter NBDs (3, 4). Thus, the Walker A consensus sequence (5), also termed P-loop, engages two nucleotide binding patterns across intracellular or cell surface membranes (1). All ABC transporters possess two transmembrane domains, each generally composed of six membrane-spanning segments, and two nucleotide binding domains (NBDs) with primary sequence homology across the protein family. Whereas substrate interaction is generally thought to involve the transmembrane domains, the NBDs bind and hydrolyze ATP. Both of the latter events have been shown to lead to conformational changes that upon transmission to substrate binding domains in an undefined fashion result in substrate translocation (2).

Several models have been formulated that link ATP metabolism to substrate transport (2). These models generally postulate that, depending on the nature of the bound nucleotide, NBDs can assume at least three states: an ATP-associated state, followed by a short-lived transition state with ADP and inorganic phosphate (P_i) bound, and an ADP-bound state. Transition from one state to the next is thought to be coupled to re-orientation of the corresponding substrate binding site and/or a change in substrate affinity. ATP metabolism by the two NBDs of an ABC transporter is generally believed to be linked in an allosteric fashion, as mutations in one NBD affect ATP processing by the other NBD, at least in most cases. Indirect evidence suggests that ATP metabolism by some ABC transporters proceeds in an alternating fashion, such that only one NBD at a time hydrolyzes ATP. This conclusion is mainly based on “vanadate trapping” experiments in which only one of the two NBDs of P-glycoprotein (P-gp), a drug efflux pump overexpressed in many tumors, can be “frozen” in the ADP–P_i bound transition state (6). Recent studies suggest that LmrA, a bacterial analog of P-gp, also uses a “two cylinder engine” model and elegantly provide evidence on how orientation and affinity of the (in this case presumably two) substrate binding sites change during the catalytic cycle (7). Whereas the P-gp NBDs are thought to metabolize ATP in an alternating but otherwise identical fashion (8), recent evidence suggests that other ABC transporters may have assigned distinct roles to the...
two NBds. Thus, the multidrug resistance protein (MRP1), an organic anion exporter also transporting many anti-cancer drugs, appears to hydrolyze ATP preferentially or initially after substrate binding at its NBd2 (9–11). Among several interpretations of these non-equivalent roles of the MRP1 NBds, the hypothesis that nucleotide binding to NBd1 may regulate nucleotide hydrolysis by NBd2 is noteworthy (9).

TAP1 and TAP2 form a heterodimeric complex in the membrane of the endoplasmic reticulum and proximal Golgi that provides antigenic peptides to newly synthesized and assembling MHC class I molecules (12). TAP is essential for MHC class I-restricted antigen presentation, as demonstrated by absent or low MHC class I expression in TAP-deficient mice and cell lines (13). Tumor cell lines frequently show impaired TAP expression and/or function, underlining the key role of TAP in MHC class I-mediated immune surveillance and prevention of tumor growth (14). Several herpes virus proteins block TAP function, presumably through different mechanisms involving conformational change (15). Thus, diminution or loss of TAP function plays a role in the pathogenesis of viral and malignant diseases. These perturbations of TAP function may be related to mutations or protein interactions interfering with ATP metabolism; indeed, a case of a human lung tumor with impaired TAP function because of a mutation in the Walker B region has been described (16).

Little is known on ATP metabolism by the TAP transporters. ATP hydrolysis is required for peptide transport by TAP (17). We have recently described that at 37 °C, microsomal TAP requires stabilization by nucleotide di- or triphosphates to maintain a conformation with high substrate affinity (18). Similarly, Knittler et al. (19) reported that substitutions in the TAP Walker A sequences not only abolished peptide binding to, and transport by, TAP, but also inhibited the physiological dissociation of peptide-assembled MHC class I molecules from TAP. In this study, we used an insect cell expression system to study substrate interaction with TAP proteins carrying mutations in Walker A and B sequences. Our results shed light on the nucleotide requirements for TAP folding and for peptide binding and transport and suggest that the NBds of TAP1 and TAP2 fulfill distinct functions in the catalytic TAP cycle.

**EXPERIMENTAL PROCEDURES**

**Mutant TAP Proteins—** Baculoviruses encoding human wild type (wt) TAP1.A and TAP2.A proteins with mutations in the Walker sequences were produced by oligonucleotide-directed “loop out” mutagenesis on uracil-enriched single-stranded DNA as described previously (20). cDNAs containing the desired mutations, confirmed by sequencing, were cloned into the baculovirus transfer vector pVL1392 (Invitrogen, San Diego, CA), and co-transfected with BaculoGold™ (PharMingen, San Diego, CA) virus DNA into Sf9 insect cells to produce recombinant viruses as described previously (20).

**Production of Microsomes—** Insect cell microsomes expressing mutant TAP proteins were produced by sucrose gradient fractionation of mechanically lysed infected Sf9 cells according to previously published procedures (21). A mixture of protease inhibitors was added before insect cell lysis (chymostatin, phenylmethylsulfonyl fluoride, pepstatin, apronin, and leupeptin; see Ref. 20 for details).

**Peptide Binding and Transport Assays—** TAP function was evaluated in peptide binding and transport assays performed exactly as described previously, using [3H]-labeled reporter peptides R-9L (RYRNNASTEL) and R-10-T (RYWANATRST) for binding and transport assays, respectively (20). In preincubation experiments, microsomes (15–30 μl) in 150 μl of phosphate-buffered saline with 0.1 mg/ml bovine serum albumin, 1 mM dithiothreitol, and 2 mM MgCl2 were incubated for 15 min at 37 °C, cooled to 4 °C in a water/ice bath, pelleted (20,000 × g for 5 min), resuspended in 150 μl of assay buffer, and used in a standard binding assay.

**Immunoprecipitation, Western Blot, and Flow Cytometric Analysis—** These experiments were also performed as described previously by us (18, 22). For immunoprecipitation, 30-μl (for vesicles prepared up to three months prior to the experiment) or 60-μl microsomes (for older vesicles) were lysed in a total volume of 600 μl using Nonidet P-40, and TAP complexes were immunoprecipitated with monoclonal antibody (mAb) 148.3 specific for human TAP1 (23). Proteins blotted onto polyvinyldene difluoride membranes were visualized with mAbs 148.3 or 429.3 (anti-TAP2) and a standard enhanced chemiluminescence protocol. For flow cytometric analysis, Sf9 cells were infected with TAP1 and TAP2 viruses, both at a multiplicity of infection of 6, together with a virus encoding HLA-B27 and human β2-microglobulin at a multiplicity of infection of 3. Viruses coding for two unrelated proteins, 65-kDa glutamic acid decarboxylase (GAD), and IA-2, the intracellular fragment of a tyrosine phosphatase, were used in control infections for cytometric analysis (24). Cells were harvested 40 h after infection and stained for cell surface expression of HLA-B27 with mAbs B27.1.M1 and W6/32. Dead cells staining with propidium iodide were excluded from analysis.

**ATP Binding Assay—** 5 × 106 Sf9 cells were infected with one or two TAP viruses, each at a multiplicity of infection of 10 and incubated for 72 h before harvesting. Pelleted cells were lysed for 40 min at 4 °C in 750 μl of a 10 mM Tris, pH 7.4 buffer containing 10 mM sodium phosphate, 130 mM NaCl, 5 mM MgCl2, and 1% Triton X-100. After lysate clarification by centrifugation (40,000 × g, 30 min), 75–250 μl of lystate (corresponding to 0.5–1.5 × 106 cells) was diluted to 300 μl and incubated for 2 h at 4 °C with 50 μl of packed ATP-analogous beads (11 atom spacer, Sigma-Aldrich, St.-Quentin, France) in the presence or absence of ATP or ADP. Then beads were washed three times with 1 ml of the same buffer, with 0.5% Triton X-100, and finally resuspended in 30 μl of SDS-PAGE sample buffer. 7 μl were then loaded on 7.5% SDS-PAGE gels, and precipitated TAP proteins were detected by Western blot analysis using mAb 148.3 and 429.3, as described above.

**RESULTS**

**Insect Cell Expression of Mutant TAP Proteins—** To study the role of nucleotides for TAP function, TAP proteins with mutant Walker sequences were designed and expressed in the Sf9 insect cell/baculovirus system. Mutants included single residue substitutions as well as complete replacement or deletion of Walker A sequences (Table I). Point substitutions in the Walker A motif were designed according to published studies. Substitution of the highly conserved Lys residue (mutants T1K544A and T2K509A), and of the amino-terminal Gly residues (mutants T1K544A and T2K509A), and of the amino-terminal Gly residues (mutants T1G538Q, T1S545/6TS, and T2S510/1TS) has been reported to enhance efficiency of MgATP utilization by the cystic fibrosis transmembrane conductance regulator (CFTR) (25). Finally, mutation T1R659Q corresponds to a natural mutation in the TAP1 Walker B motif previously identified in a human small cell lung cancer line and was described to be associated with a TAP-deficient cell phenotype (16). Replacements of entire Walker A motifs by synthetic linker peptides (mutants T1Rep and T2Rep), or complete Walker A deletions (mutants T1Del and T2Del) were included to study TAP proteins highly likely to be unable to hydrolyze and bind ATP.

Recombinant baculoviruses containing mutant TAP cDNAs
were generated, and expression and assembly of TAP proteins were studied in Western blotting and immunoprecipitation experiments (Fig. 1). All mutant TAP1 and TAP2 proteins were expressed and migrated as expected in SDS-PAGE gels. However, whereas most mutant TAP subunits were overexpressed to a similar extent as wild-type TAP1 and TAP2, only small amounts of mutant TAP1 subunit T1Del could be immunoprecipitated, and recovery of mutant T1G538Q was also reduced. Reduced levels of immunoprecipitable T1Del and T1G538Q proteins reflected reduced levels of mutant proteins in microsome lysates prepared using Nonidet P-40 (see below); however, relatively large amounts of these subunits were detected in microsome “debris” that could not be solubilized in Nonidet P-40 (not shown). This suggested that mutants T1Del and T1G538Q folded inefficiently and formed insoluble aggregates. Nevertheless, even in these cases, immunoprecipitable material, presumably corresponding to the correctly folded fractions, assembled efficiently in heteromeric TAP complexes, as indicated by co-immunoprecipitation (Fig. 1). Thus, none of the mutations had an adverse effect on assembly with wild-type complementary subunits.

**Peptide Translocation and Supply to HLA-B27 Molecules by Mutant TAP Complexes**—To analyze the effect of the mutations on TAP transport function, insect cells expressing mutant TAP subunits together with wild-type complementary subunits were produced and tested in two assays. One of these evaluates accumulation of a glycosylated radiolabeled peptide in microsomeosomes prepared using Nonidet P-40 (see below); however, relatively large amounts of these subunits were detected in microsome “debris” that could not be solubilized in Nonidet P-40 (not shown). This suggested that mutants T1Del and T1G538Q folded inefficiently and formed insoluble aggregates. Nevertheless, even in these cases, immunoprecipitable material, presumably corresponding to the correctly folded fractions, assembled efficiently in heteromeric TAP complexes, as indicated by co-immunoprecipitation (Fig. 1). Thus, none of the mutations had an adverse effect on assembly with wild-type complementary subunits.

![Image](http://www.jbc.org/)

**FIG. 1.** Recovery by immunoprecipitation and complex formation of mutant TAP proteins. 60 µl of microsome solution were lysed in a buffer containing 1% Nonidet P-40, and TAP complexes were precipitated from cleared lysates with mAb 148.3 that is specific for TAP1. Equal parts of the immunoprecipitated material were separated in two 7.5% SDS-PAGE gels, blotted on polyvinylidene difluoride membranes, and stained with mAb 148.3 (upper panels) or 429.3 specific for TAP2 (bottom panels). Mutant or wild-type (wt) TAP proteins expressed by microsomes are indicated above or below the panels, respectively. In this and the following figures, inversion mutants (T1ST545/6TS and T2ST510/1TS) are designated Co (for control).

![Image](http://www.jbc.org/)

**FIG. 2.** Peptide transport by mutant TAP complexes. Accumulation of glycosylated peptide R-10-T in insect cell microsomes expressing the indicated TAPI/TAP2 combinations was measured after incubation at 37 °C for the time indicated. ATP and ADP were used at 1 mM concentrations.

| Condition | Time (min) | 1w/2wt | 1Rep/2wt | 1Del/2wt | 1K544A/2wt | 1G538Q/2wt | 1R659Q/2wt | 1w2/2Del | 1w2/K509A | 1w2/Co |
|-----------|-----------|--------|----------|----------|-----------|-----------|-----------|---------|----------|--------|
| Buffer    | 0         | 100    | 100      | 100      | 100       | 100       | 100       | 100     | 100      | 100    |
| ATP       | 0         | 100    | 100      | 100      | 100       | 100       | 100       | 100     | 100      | 100    |
| ADP       | 0         | 100    | 100      | 100      | 100       | 100       | 100       | 100     | 100      | 100    |

As expected, substitutions T1K544A, T1G538Q, and T2K509A, as well as Walker A replacements and deletions in either TAP subunit, abolished accumulation of glycosylated peptide in the ER, whereas control mutation T2ST510/1TS did not affect the rate of peptide accumulation and T1ST545/6TS increased it (Fig. 2). Thus, ATP binding to and/or hydrolysis by both NBDs is required for peptide transport by TAP. Moreover, similar to the CFTR (25), mutation T1ST545/6TS may enhance efficiency of ATP hydrolysis by TAP1. Surprisingly, mutation T1R659Q, reported to create a TAP-deficient cell phenotype (16), reduced peptide accumulation by about 50% rather than abolishing it. ATP-independent peptide accumulation after prolonged microsome incubation at 37 °C is likely to reflect vesicle “leakiness.” ADP, which is known to act as a competitor for ATP in ABC transporters (25), reduced peptide accumulation in microsomes expressing wild-type TAP or control mutations (ST to TS inversion) to levels below those observed without addition of nucleotides. This may indicate that ADP competed with ATP carried over in microsome purification for binding to the TAP NBDs.

Analysis of HLA-B27 expression confirmed results obtained in transport assays (Fig. 3). In accordance with our previous observation (22), B27 was not detectable on the surface of insect cells expressing HLAB27/β2-m together with the two unrelated control proteins GAD and IA-2 (mean fluorescence 5). Co-expression of wild-type TAP complexes induced B27 expression by about 50% of the cells (mean 20). Expression was limited to 50% because cells were infected with a low multiplicity of infection of the B27/β2-m virus together with a high multiplicity of infection of TAP viruses to ensure TAP expression by all B27-expressing cells. Whereas control mutations (ST to TS inversions) increased B27 expression to the same extent as wild-type TAP (means of 20), TAP mutants abolishing peptide transport had no significant effect on its cell surface density (means between 6 and 7). Importantly, mutation
T1R659Q displayed about 50% of the effect of wild-type TAP (mean 14). Thus, TAP-mediated peptide supply to HLA-B27 also required ATP binding to and/or hydrolysis by both TAP subunits and was reduced but not abolished by the Walker B mutation described to eliminate HLA class I expression on the surface of a tumor cell line.

Peptide Binding to Mutant TAP Complexes at Low Temperature and 37 °C—Peptide transport is preceded by a high affinity interaction of peptide with the TAP substrate binding site, which can be measured in a binding assay performed at low temperature (21). We have previously shown that wild-type TAP complexes incubated at 37 °C in the absence of nucleotide di- or triphosphates change conformation and lose the ability to bind peptide with high affinity (18). We have therefore proposed that low TAP affinity for peptide substrate characterizes a "nucleotide-off" conformation that may be related albeit not identical to a transition state associated with peptide release during the transport cycle. Here we asked how Walker motif mutations impairing nucleotide binding and/or hydrolysis affect peptide binding to TAP at different temperatures.

In standard peptide binding assays with TAP-expressing vesicles not exposed to temperatures above 27 °C (the temperature used for insect cell culture), microsomes expressing TAP complexes with deletion of the Walker A sequence in either TAP subunit consistently showed very low but significant peptide binding. This binding exceeded binding to control vesicles expressing TAP1 by a factor of 10–15 (Fig. 4). Microsomes expressing mutant T1Rep also consistently displayed significantly reduced peptide binding capacity. Peptide binding to mutant T2Rep was moderately reduced, whereas other TAP1 mutants, including those with Walker A replacement, bound normal peptide amounts.

When microsomes were incubated for 15 min at 37 °C prior to binding assays, all TAP complexes lost binding capacity (Fig. 5). This included complexes composed of two mutant subunits, for example T1Rep/T2Rep or T1K544A/T2K509A (not shown). Thus, ATP hydrolysis was not required for loss of high affinity peptide binding by TAP. Addition of ATP or ADP during incubations at 37 °C preserved peptide binding capacity by some but not all TAP complexes containing mutated subunits. TAP1 mutations T1Del and T1G538Q abolished TAP complex stabilization by ATP. Complexes containing other TAP1 mutations, including mutation T1Rep, retained peptide binding capacity in the presence of ATP or ADP to a similar extent as wild-type TAP complexes. However, complexes with similar (T2Rep) or identical (T2K509A) mutations in TAP2 did not retain peptide binding capacity in the presence of nucleotides. This suggested that binding of nucleotide di- or triphosphates to TAP2 is essential for maintaining TAP peptide binding capacity whereas nucleotide binding to TAP1 is not. Moreover, TAP1 mutants prone to aggregation also could not be stabilized by nucleotides.

Nucleotide Binding Affinity of Mutant TAP Subunits—To provide direct evidence for the role of nucleotide binding, as opposed to hydrolysis, for peptide binding to TAP, we measured the binding affinity of mutant TAP subunits for ATP immobilized on agarose beads (Fig. 6). Initially, we studied ATP bind-
Mutational Analysis of TAP Walker Sequences

FIG. 6. Binding of mutant TAP proteins to immobilized ATP. Cells expressing individual TAP subunits or combinations thereof were lysed in a buffer containing Triton X-100, and TAP proteins binding ATP were recovered by incubation with ATP-agarose beads. TAP proteins binding to the beads, and in parallel total solubilized TAP proteins, were visualized in Western blots stained with mAb 148.3 or 429.3. In A, Triton X-100-solubilized total TAP proteins in cells expressing (top to bottom) TAP1 only, TAP2 only, or both wild-type subunits are shown on the left. TAP proteins recovered from 2.3 × 10^6 cells by incubation with ATP-agarose, in the presence of the nucleotides indicated under the panels, are shown on the right. In B, the top panel shows total TAP1 proteins recovered from cells expressing isolated mutant TAP1 subunits, whereas the lower panel shows ATP-binding TAP1 proteins recovered from the same cells (1.2 × 10^6 cell equivalents/lane). In C, cells expressing TAP1 mutant K544A together with indicated mutant TAP2 proteins. Total solubilized TAP2 protein and ATP-binding TAP2 protein corresponding to 4 × 10^5 cell equivalents are shown in the top and bottom panels, respectively. TAP1 protein expression was identical in all samples (data not shown).

Binding of isolated or co-expressed wild-type TAP subunits (Fig. 6A). ATP binding of isolated wild-type TAP1 was efficient and inhibited with equal efficiency by ADP and ATP but not AMP. In contrast, isolated TAP2 subunits showed relatively low levels of Nonidet P-40 solubilized material, and ATP binding was hardly detectable. This suggested that isolated TAP2 folded poorly and that correctly folded isolated TAP2 bound ATP poorly. Co-expression of the two wild-type subunits had no effect on the efficiency of TAP1 binding to ATP but induced a significant increase in the amount of Nonidet P-40 solubilized TAP2, suggesting that correct TAP2 folding is enhanced in the presence of TAP1. Simultaneously, the amount of TAP2 recovered by incubation with ATP-agarose increased dramatically and by a factor largely exceeding the effect of TAP1 on TAP2 folding (Fig. 6A). The latter phenomenon indicated either indirect recovery of TAP2 (itself with low ATP affinity) via ATP-bound TAP1, or an increase in the ATP binding affinity of TAP1-associated TAP2.

Because isolated TAP1 subunits can bind ATP and be stabilized by it, ATP binding affinities of TAP1 mutants were first studied in the absence of TAP2 (Fig. 6B). Western blot analysis of Nonidet P-40-solubilized material confirmed low expression levels of Nonidet P-40-solubilized T1Del and T1G538Q mutants, whereas all other mutants, including T1Rep, were expressed at similar high levels. Control mutation T1ST545/6TS and Walker B mutation T1R659Q had no effect on ATP binding affinity, whereas all other TAP1 mutations abolished ATP binding completely. Identical results were obtained with cells co-expressing TAP1 mutants with wild-type TAP2 (not shown). The latter experiments also demonstrated that complex formation with TAP1 does not increase the ATP binding affinity of TAP2. Co-expression of T1K544A (being itself unable to bind ATP) increased the amount of Nonidet P-40-solubilized TAP2 as efficiently as wild-type TAP1; however, TAP2 binding to ATP remained very low in its presence (not shown).

Because of the poor folding and ATP binding affinity (Fig. 6A), isolated mutant TAP2 proteins could not be used for ATP binding experiments. To increase the amount of correctly folded TAP2 material, we chose to co-express a mutant TAP1 protein (T1K544A) that itself could not bind to immobilized ATP (Fig. 6B). Moreover, to compensate for inefficient binding of TAP2 to ATP-agarose, experiments were performed on a 4-fold higher amount of cell lysate. Western blot analysis of Nonidet P-40 lysates confirmed that, among TAP2 mutants, only T2Del showed slightly reduced expression levels, possibly because of reduced folding efficiency (Fig. 6C). Using relatively large amounts of cell lysate, efficient binding of T2wt to ATP-agarose was detected. Control mutant T2ST510/1TS also bound ATP, whereas the other three mutants completely lacked ATP binding. Thus, both in TAP1 and TAP2, substitutions in as well as replacement and deletion of the Walker A motifs completely abolished ATP binding.

Conformational Changes in Mutant TAP Complexes at 37 °C—We have previously shown that loss of peptide binding capacity by TAP at 37 °C is associated with a conformational change that reduces TAP recognition by several antibodies to TAP1 and TAP2 (18). Because peptide binding to TAP complexes containing mutant TAP1 subunits unable to bind nucleotides was preserved by ATP, we wondered whether this reflected a dissociation between TAP1 conformation and TAP peptide binding capacity. An alternative explanation was that...
nucleotide binding to TAP2 might stabilize TAP1 indirectly. We therefore precipitated TAP complexes that had been incubated at 4 or 37 °C for 15 min in the presence or absence of ATP with antibodies to TAP1 and determined the efficiency of precipitation by Western blot analysis. These experiments exploit the phenomenon that incubation of TAP at 37 °C in the absence of nucleotides induces a conformational change that reduces recognition of native Nonidet P-40-solubilized TAP1 by mAb 148.3 without affecting its recognition in a denatured state, i.e. in Western blots. Because wild-type TAP1 subunits can bind and be stabilized by ATP whereas TAP2 subunits cannot (18), experiments were also performed with microsomes expressing TAP1 only.

Similar to wild-type TAP complexes, incubation of isolated mutant TAP1 subunits or TAP complexes containing such subunits at 37 °C leads to a strong decrease in the amount of recovered TAP proteins (Fig. 7 and data not shown). TAP1 mutations T1K544A, T1G538Q, and T1Rep abrogated stabilization of the isolated TAP1 subunit whereas mutations T1ST545/6TS and T1R659Q did not (Fig. 7 and Table II). However, dimeric TAP complexes containing mutants T1K544A and T1Rep were stabilized efficiently by ATP, which could only be due to ATP binding to TAP2. ATP did not stabilize mutant TAP2 subunits expressed in complexes with wild-type TAP1 or complexes containing mutant T1G538Q (Table II). Taken together, these experiments demonstrate that peptide binding capacity of complexes containing mutant TAP subunits is closely associated with conformational changes. Like peptide binding capacity, TAP conformation is maintained by nucleotide binding to the TAP2 subunit, which controls the conformation of the entire TAP complex and stabilizes TAP1 in an indirect manner.

**DISCUSSION**

The mutants produced in this study provide insight into the regulation of TAP function by nucleotides. We find that the Walker sequences of the two TAP subunits have distinct roles and affect TAP structure as well as peptide binding and transport. Whereas the TAP1 NBD appears to be important for TAP1 folding and overall complex stability, the TAP2 NBD controls substrate affinity and more limited conformational changes.

Analysis of mutant expression, solubility in Nonidet P-40, and immunoprecipitation provided some insight into the mechanism of TAP folding and assembly. In the case of TAP1, not only deletion of the Walker A sequence, but also point mutation T1G538Q strongly decreased the amount of Nonidet P-40 soluble protein, suggesting that TAP1 folding is influenced by its NBD. However, in the case of mutants T1K544A and remarkably also T1Rep, high expression levels and normal peptide binding capacity at 4 °C and 37 °C argue against an effect on folding. Whereas folding of the isolated TAP2 subunit is much less efficient than that of TAP1, mutants T2K509A and T2Rep also have no detectable effect on folding and even deletion of its Walker A motif affects folding moderately. Taken together, these observations suggest a model in which assembly of TAP complexes proceeds via initial folding of the TAP1 subunit, possibly bound to nucleotides, which in turn serves as a scaffold supporting TAP2 folding and simultaneous dimer formation.

ATP binding assays provided clear evidence that substitution of the Lys residue in the Walker A sequence, which engages in extensive interactions with the β-phosphate of bound ATP in HisP (4), or replacement of the entire sequence, abolishes nucleotide binding to TAP1 and TAP2. Thus, TAP differs from other ABC transporters such as P-gp in which Lys substitution has been reported to affect ATP hydrolysis but not binding (27). ATP binding assays also suggested that TAP2, expressed as an isolated subunit or assembled with TAP1, has lower ATP binding affinity than TAP1. Finally, comparison of these assays with our “nucleotide stabilization assay,” in which TAP is immunoprecipitated after incubation at 37 °C, demonstrates that the latter assay reflects nucleotide binding and can replace ATP binding assays. Mutation T1R659Q is the only one without effect on ATP binding and TAP1 stabilization at 37 °C. It is therefore likely to selectively impair ATP hydrolysis, a conclusion in agreement with the proposed role of the Walker B residues in coordinating MgATP hydrolysis (3, 4).

Not surprisingly, we find that all mutations abolishing nucleotide binding to either TAP subunit eliminate peptide transport by TAP, demonstrating that nucleotide interaction of both subunits is required for transport. This observation is in agreement with a previous study by Knittler and associates (19). However, to our knowledge, substitution T1R659Q is the first case to be studied of a TAP mutation likely to exclusively affect ATP hydrolysis. This mutation reduced TAP transport by only 50%. Impairment rather than abolition of TAP function evidently may reflect reduced but not absent ATP hydrolysis. An interesting alternative interpretation can be formulated with reference to other ABC transporters such as MRP1. In that case, NBD1 has been proposed to regulate NBD2, thereby explaining a complete functional knockout by mutation of NBD2 in contrast to 70% loss of function only by mutation of NBD1 (9). Elucidation of the role of ATP hydrolysis by TAP1 and TAP2 will require an assay measuring ATP hydrolysis by TAP, which is as yet unavailable. It is important to underline that mutations impairing exclusively ATP hydrolysis by TAP2 have not been reported and studied so far, so that a requirement for ATP hydrolysis by TAP2 remains hypothetical.

Whatever the reason for the limited effect of the T1R659Q mutation on TAP transport and B27 expression, a 50% function by T1R659Q mutant TAP proteins is a surprising finding, given that the human small cell lung cancer line H1436, in which the mutation was found, was described as essentially “HLA class I negative” (16). Several explanations may account for this discrepancy. It appears unlikely that H1436 harbors additional defects in antigen presentation genes as TAP1 transfection restored class I expression (16). However, Chen et al. (16) showed that H1436 expresses low TAP1 levels and that expression of high levels of T1R659Q in H1436 resulted in significant class I expression by some, albeit not all, tumor cells. Hence, cancer cells expressing high amounts of T1R659Q may resemble insect cells overexpressing it, and the phenotype of H1436 may be caused by a combination of low TAP expression and impaired TAP function. It is also conceivable that mutation T1R659Q affects TAP interaction with class I loading complexes, for example by inhibiting release of peptide-loaded class I molecules (19) or by impairing tapasin interaction (29). In any
case, it is interesting to note that a 50% reduction in TAP function can result in loss of class I expression. TAP may act as a factor limiting antigen presentation by non-lymphoid cells and is therefore an especially “promising” target for mutations or regulatory proteins that allow for tumor or viral escape from immune surveillance (15, 30).

This study clearly demonstrates that peptide binding to TAP complexes, i.e., peptide affinity, is controlled by nucleotide interaction with TAP2 but not TAP1. Our results suggest that the TAP substrate binding site can assume at least two conformations distinguished by their peptide affinities. The low affinity state can be produced by three experimental manipulations: (i) incubation of microsomal wild-type TAP complexes at 37°C in the absence of nucleotides, (ii) incubation at 37°C in the presence or absence of nucleotides, of TAP complexes with substitutions in the TAP2 Walker A sequence abolishing nucleotide binding, and (iii) deletion of the Walker A sequence in TAP2. In other words, low peptide affinity can either be induced in a temperature-independent fashion by deletion of the TAP2 Walker A sequence or at 37°C by absence of nucleotide binding to it. In contrast, reduced peptide binding to T1Del/T2wt and T1G538Q/T2wt complexes reflects a reduced amount of correctly folded TAP complexes formed by these mutants rather than a conformation with low peptide affinity. The amount of peptide bound by these complexes corresponds to the amount of immunoprecipitable and presumably correctly folded complexes (Figs. 1 and 4). Absence of nucleotide stabilization of these complexes at 37°C may then indicate that nucleotide binding to TAP2 cannot prevent aggregation of these presumably unstable TAP1 subunits. At 37°C, complexes with replacements of both Walker A sequences (T1Rep/T2Rep) undergo the same conformational change associated with loss of peptide binding capacity as wild-type TAP complexes. Thus, transition to the TAP state with low peptide affinity can take place in the absence of nucleotide interaction and appears to reflect a spontaneous conformational change at 37°C that is reversed or prevented by nucleotide binding to TAP2.

Nucleotide binding to TAP2 not only controls TAP peptide affinity but also is sufficient to prevent a substantial conformational change of TAP complexes at 37°C. It is important to underline that this change is different from that induced by mutations T1Del and T1G538Q. Whereas the mutations lead to formation of aggregates that are insoluble in Nonidet P-40, incubation at 37°C does not. Thus, while TAP1 serves as a scaffold that folds under the control of its NBD and permits secondary folding and association of TAP2, nucleotide binding to TAP2 induces a distinct conformation with high peptide affinity. In controlling substrate affinity by nucleotide interaction with a single subunit, the TAP complex differs from some extensively studied ABC transporters such as P-gp that possess two functionally identical NBDs (6). However, TAP may resemble other ABC transporters such as CFTR or MRP1 in which the two NBDs seem to have distinct functions (6, 9, 25).

TAP also appears to differ from other ABC transporters with respect to the nucleotides bound in the state with high substrate affinity. Whereas other transporters display low substrate affinity (required for substrate release) when in the ADP-bound state (2, 7), ADP is clearly indistinguishable from ATP with respect to preserving high peptide binding affinity of TAP. Thus, TAP complexes may release peptide either after complete nucleotide dissociation or during the transition state associated with ADP and P_i. In contrast to P-gp, which can be frozen in the latter state by incubation with orthovanadate and ATP (31), an experimental technique for inducing the transitional conformation in TAP has not been described. However, our observation that low peptide affinity can be induced by incubation at 37°C of TAP complexes unable to bind nucleotides argues for a nucleotide-off state as an intermediate with low substrate affinity. Our data suggest that this state is followed by nucleotide binding to TAP2, which allows TAP complexes to acquire a high peptide affinity and initiate a new transport cycle.

REFERENCES
1. Higgins, C. F. (1992) Annu. Rev. Cell Biol. 8, 67–113
2. Schneider, E., and Hunke, S. (1998) FEMS Microbiol. Rev. 22, 1–20
3. Diederichs, K., Diez, J., Greller, G., Muller, C., Bredel, J., Schnell, C., Vonrnein, C., Boos, W., and Welte, W. (2000) EMBO J. 19, 5951–5961
4. Hung, L. W., Wang, I. X., Nakaide, K., Liu, P. Q., Ames, G. F., and Kim, S. H. (1998) Nature 396, 705–707
5. Walker, J. E., Suruste, M., Bunswick, M. J., and Gay, N. J. (1982) EMBO J. 1, 945–951
6. Senior, A. E., and Gadesby, D. C. (1997) Semin. Cancer Biol. 8, 143–150
7. van Veen, H. W., Margolles, A., Muller, M., Higgins, C. F., and Konings, W. N. (2000) EMBO J. 19, 2503–2514
8. Senior, A. E., al-Shawi, M. K., and Urbatsch, I. L. (1995) FEBS Lett. 377, 285–289
9. Gao, M., Cui, H. R., Lee, D. W., Grant, C. E., Altoquist, K. C., Cole, S. P., and Deeley, R. G. (2000) J. Biol. Chem. 275, 13098–13108
10. Hou, Y., Cui, L., Rijordan, R. J., and Chang, X. (2000) J. Biol. Chem. 275, 20280–20287
11. Nagata, K., Nishitani, M., Matsuo, M., Kioka, N., Amachi, T., and Ueda, K. (2000) J. Biol. Chem. 275, 17626–17630
12. Elliott, T. (1997) Adv. Immunol. 63, 47–109
13. Spiess, T., and Demars, B. (1999) Nature 351, 323–324
14. Algarza, I., Cabrera, T., and Garrido, F. (2000) Hum. Immunol. 61, 65–73
15. Tortorella, D., Gewurz, B. E., Furman, M. H., Schust, D. L., and Ploegh, H. L. (2000) Annu. Rev. Immunol. 18, 861–926
16. Chen, H. L., Grubrlovič, D., Tampe, R., Girgis, K. R., Nadaf, S., and Carbone, D. P. (1996) Nat. Genet. 13, 210–213
17. Neefjes, J. J., Momburg, F., and Hammerling, G. J. (1993) Science 261, 769–771
18. van Endert, P. M. (1999) J. Biol. Chem. 274, 14632–14638
19. Knittler, M. R., Alberts, P., Deverson, E. V., and Howard, J. C. (1999)Curr. Biol. 9, 999–1008
20. Daniel, S., Caillat-Zuecm, S., Hammer, J., Bach, J. F., and van Endert, P. M. (1997) J. Immunol. 158, 2350–2357
21. van Endert, P. M., Tampe, R., Meyer, T. H., Tisch, R., Bach, J. F., and McDewitt, H. O. (1994) Immunity 1, 491–500
22. Lavaux, G., Gubler, B., Cohen, H., Daniel, S., Caillat-Zucman, S., and van Endert, P. M. (1999) J. Biol. Chem. 274, 31349–31358
23. Mery, M. H., van Endert, P. M., Uebel, S., Ehring, B., and Tampe, R. (1994)FEBS Lett. 351, 443–447
24. Bach, J. M., Otto, H., Neppem, G. T., Jung, G., Cohen, H., Timis, J., Buitard, C., and van Endert, P. M. (1997) J. Autoimmun. 10, 375–386
25. Anderson, M. P., and Welsh, M. J. (1992) Science 257, 1701–1704
26. Szabo, K., Welker, E., Bakos, Muller, M., Roninson, I., Varadi, A., and Sarkadi, B. (1998) J. Biol. Chem. 273, 10132–10138
27. Urbatsch, I. L., Gimi, K., Wilke-Mounts, S., and Senior, A. E. (2000) J. Biol. Chem. 275, 25031–25038
28. Daniel, S., Bruscic, V., Caillat-Zuecm, S., Petrovsky, N., Harrison, L., Riganelli, D., Sinigaglia, F., Gallazzi, F., Hammer, J., and van Endert, P. M. (1998) J. Immunol. 161, 617–624
29. Cresswell, P., Bangia, N., Dick, T., and Diedrich, G. (1999)Immunol. Rev. 172, 21–28
30. Garrido, F., Ruiz-Cabello, F., Cabrera, T., Perez-Villar, J. J., Lopez-Botet, M., Duggle-Keen, M., and Stern, P. L. (1997)Immunol. Today 18, 89–95.
31. Urbatsch, I. L., Sankaran, B., Weber, J., and Senior, A. E. (1995) J. Biol. Chem. 270, 13933–13939
Distinct Functions of the ATP Binding Cassettes of Transporters Associated with Antigen Processing: A MUTATIONAL ANALYSIS OF WALKER A AND B SEQUENCES

Loredana Saveanu, Soizic Daniel and Peter M. van Endert

J. Biol. Chem. 2001, 276:22107-22113.
doi: 10.1074/jbc.M011221200 originally published online April 4, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M011221200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 31 references, 14 of which can be accessed free at http://www.jbc.org/content/276/25/22107.full.html#ref-list-1