Role of Nucleotides and Peptide Substrate for Stability and Functional State of the Human ABC Family Transporters Associated with Antigen Processing*

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The transporters associated with antigen processing (TAP) belong to the family of ATP-binding cassette (ABC) transporters which share structural organization and use energy provided by ATP to translocate a large variety of solutes across cellular membranes. TAP is thought to hydrolyze ATP in order to deliver peptides to the endoplasmic reticulum where they can assemble with major histocompatibility complex class I molecules. However, initial binding of peptide substrates to TAP has been suggested to be ATP-independent. In this study, the effect of temperature, energetic nucleotides, and peptide on conformation and functional capacity of TAP proteins was examined. Incubation of insect cell microsomes overexpressing human TAP complexes or of human B cell microsomes at 37 °C induced a rapid and irreversible structural change that reduced dramatically TAP reactivity with antibodies to transmembrane and nucleotide-binding domains and abolished peptide binding and transport by TAP. These alterations were inhibited almost completely by di- or trinucleotides, and partially by high affinity peptides, suggesting that complete nucleotide dissociation inactivates TAP complexes. Experiments with isolated TAP subunits and fragments suggested that TAP complex stabilization by nucleotides may depend on their binding to the TAP1 subunit. Thus, the cellular level of functional TAP complexes may be regulated by nucleotide concentrations. It is speculated that this regulation may serve to prevent induction of autoimmunity by stressed cells with low energy levels.

ABC transporters constitute a large protein family whose members use energy provided by ATP to transport a large array of solutes across cellular membranes or, in a few cases, to regulate opening of membrane channels (reviewed in Ref. 1). ABC transporters are composed of four domains, two hydrophilic nucleotide-binding domains (NBD), and two hydrophobic domains each comprising six or more transmembrane segments. These domains can be encoded by between one and four separate genes. While the hydrophobic transmembrane domains of individual ABC proteins share little primary sequence homology, the nucleotide-binding domains comprise several highly conserved sequence motifs (Walker A and B) found in many ATPases and involved in binding of MgATP, and an additional “signature” sequence typical for members of the ABC family (reviewed in Ref. 2).

Although numerous studies of ABC transporters have provided strong evidence for an essential role of nucleotide binding and hydrolysis for solute transport, it remains unclear how transporter interaction with nucleotides is coupled to substrate translocation. Nucleotide binding to the hydrophilic domains has been demonstrated directly for many ABC transporters. ATP hydrolysis, although more difficult to study, has also been reported for several ABC transporters, including the cystic fibrosis transmembrane conductance regulator and the multiple drug resistance or P-glycoprotein, two ABC transporters with substantial medical importance (3, 4). Mutations affecting ATP hydrolysis by these and other ABC transporters impair or abolish their function (5–7). Studies of mutant ABC transporters, in combination with nucleotide trapping experiments using vanadate or beryllium fluoride, have given rise to several models of transporter function linking ATP binding and hydrolysis to substrate binding and release (8, 9). At least in the case of the cystic fibrosis transmembrane conductance regulator, evidence for distinct functions of the two NBD has also been obtained (5, 6).

The concept of transmembrane transport driven by ATP hydrolysis implies that transporter molecules associated with distinct nucleotide derivatives or devoid of nucleotides should display different conformations and different affinities for substrate. Evidence for the former assumption has been obtained in various systems. For example, binding of MgATP to P-glycoprotein alters its tertiary structure (10), while ATP depletion or mutational inactivation of one or both of its NBD increases reactivity with a mAb also known to inhibit its function (11). Altered conformation resulting from nucleotide binding has also been reported for several bacterial ABC transporters or their isolated NBD fragments (12, 13). Thus, ATP affects the conformation of the NBD of the bacterial maltose transporter and modulates its interaction with the hydrophobic transmembrane domains (encoded in this case by a separate gene) (14).

While the role of nucleotides for the most “prominent” mammalian ABC transporters cystic fibrosis transmembrane conductance regulator and multiple drug resistance protein has been studied in some detail, little is known about their role for the transporters associated with antigen processing, a heterodimeric complex encoded by the TAP1 and TAP2 genes in the major histocompatibility complex class II region each coding for one hydrophobic domain and one carboxyl-terminal NBD (reviewed in Ref. 15). TAP complexes transport short peptides with a length of 8 to 16 residues from the cytosol into the endoplasmic reticulum where these peptides can assemble...
with newly synthesized major histocompatibility complex class I molecules. TAP function is essential for antigen presentation by major histocompatibility complex class I molecules to cytotoxic T lymphocytes, since deficiency in one or both TAP subunits results in severely reduced major histocompatibility complex class I expression and function. As peptide transport by TAP in permeabilized cells requires ATP and is inhibited by non-hydrolyzable ATP analogues (16), TAP is thought to be an ATPase, although in experiments with isolated recombinant TAP NBD fragments, or with TAP dimers reconstituted in membranes, it has so far not been possible to provide direct evidence for this hypothesis (17–20). However, nucleotide binding to both subunits assembled in TAP complexes (21), and to the recombinant TAP1 NBD (17, 18) has been demonstrated directly; contradictory results have been reported with respect to the ATP binding capacity of the TAP2 NBD (17, 18).

TAP function involves an initial step of peptide interaction with a binding site probably composed of TAP1 and TAP2 moieties which can be monitored in human cells using photo-reactive peptides (22), or in a simple binding assay taking advantage of the substantial overexpression of TAP complexes in microsomes of insect cells infected with recombinant baculoviruses (23). In the latter system, radiolabeled peptides bind to TAP dimers with high efficiency at low temperature. Thus, in the case of TAP, solute binding to, and translocation by, the transporters can be monitored separately, providing an opportunity to study the role of nucleotides and other factors for each of these steps. Previous observations that, different from peptide transport, peptide binding to TAP does not require addition of ATP to microsomes or permeabilized cells suggested that nucleotides may not play a role for the initial substrate binding step (22, 23). However, since microsomal TAP complexes may remain ATP-associated during fractionation of subcellular compartments, and since even after depletion of cellular ATP levels the same may apply to TAP complexes in permeabilized cells, the issue of the role of nucleotides for peptide binding to TAP remains unresolved. This study was motivated by previous observations that had suggested temperature-induced conformational changes in the TAP proteins (21), and a role of nucleotides for peptide binding to TAP. Specifically, we had observed inefficient binding of peptide to TAP complexes incubated at 37 °C (23), while Russ and associates (21) reported inefficient antibody recognition of TAP complexes in lysates incubated at 37 °C. Moreover, we had previously found peptide dissociation from TAP complexes incubated with trinitrophenylated, consistent with a role for ATP in substrate binding to, and release from, TAP (23).

**EXPERIMENTAL PROCEDURES**

**Reagents**—Reporter peptides R-9L (RRYNASTEL) used in TAP binding assays, and R-10-T (RYWANATRST) used in transport assays have been described previously (23, 24). Other peptides used in Fig. 4 were a generous gift from Drs. M. Androlewicz and P. Cresswell, assays have been described previously (23, 24). Other peptides used in binding assays, and R-10-T (RYWANATRST) used in TAP transport were a generous gift from Drs. M. Androlewicz and P. Cresswell, assayed have been described previously (23, 24). Other peptides used in binding assays, and R-10-T (RYWANATRST) used in TAP transport were a generous gift from Drs. M. Androlewicz and P. Cresswell, assayed have been described previously (23, 24). Other peptides used in binding assays, and R-10-T (RYWANATRST) used in TAP transport were a generous gift from Drs. M. Androlewicz and P. Cresswell, assayed have been described previously (23, 24). Other peptides used in binding assays, and R-10-T (RYWANATRST) used in TAP transport were a generous gift from Drs. M. Androlewicz and P. Cresswell, assayed have been described previously (23, 24). Other peptides used in binding assays, and R-10-T (RYWANATRST) used in TAP transport were a generous gift from Drs. M. Androlewicz and P. Cresswell, assayed have been described previously (23, 24). Other peptides used in binding assays, and R-10-T (RYWANATRST) used in TAP transport were a generous gift from Drs. M. Androlewicz and P. Cresswell, assayed have been described previously (23, 24). Other peptides used in binding assays, and R-10-T (RYWANATRST) used in TAP transport were a generous gift from Drs. M. Androlewicz and P. Cresswell, assayed have been described previously (23, 24). Other peptides used in binding assays, and R-10-T (RYWANATRST) used in TAP transport were a generous gift from Drs. M. Androlewicz and P. Cresswell, assayed have been described previously (23, 24). Other peptides used in binding assays, and R-10-T (RYWANATRST) used in TAP transport were a generous gift from Drs. M. Androlewicz and P. Cresswell, assayed have been described previously (23, 24). Other peptides used in binding assays, and R-10-T (RYWANATRST) used in TAP transport were a generous gift from Drs. M. Androlewicz and P. Cresswell, assayed have been described previously (23, 24). Other peptides used in binding assays, and R-10-T (RYWANATRST) used in TAP transport were a generous gift from Drs. M. Androlewicz and P. Cresswell, assayed have been described previous
**RESULTS**

**Effect of Temperature on Microsomal TAP Dimers**—We had previously observed that peptide binding to insect cell-expressed human TAP dimers is less efficient when binding experiments are performed at 37 °C (23). In view of the observation by Russ and associates (21) that efficiency of TAP immunoprecipitation is reduced after incubation of detergent lysates at 37 °C, I wondered whether temperature might affect the conformation or assembly of TAP dimers expressed in microsomes, thereby affecting the functional state of TAP complexes. When microsomes expressing TAP1/TAP2 dimers were incubated under conditions employed in binding assays for 15 min at 37 °C before lysis, the amount of TAP1 protein and associated TAP2 protein precipitated by the TAP1-specific mAb 148.3 was greatly reduced (Fig. 1). TAP degradation was not responsible for lower TAP recovery, since presence of a potent mixture of protease inhibitors during preincubations did not prevent loss of precipitated TAP protein, and TAP fragments possibly resulting from degradation were not visible in Western blots (not shown). Lower TAP recovery was partly due to reduced efficiency of TAP solubilization since unseparated clarified lysates of pretreated microsomes contained smaller amounts of TAP1 and TAP2 (not shown). TAP precipitation by several antisera (especially 1p3, 1p4, and 2p4) recognizing epitopes in or between predicted TAP1 or TAP2 transmembrane domains was also diminished. However, TAP reactivity with other sera (2p1 and 2p5) was not affected by preincubation at 37 °C, and one TAP2-specific serum (2p2) precipitated exclusively heat-treated TAP dimers, albeit with low efficiency (Fig. 1). Thus, incubation of microsomes at 37 °C increased the amount of TAP proteins that could not be solubilized by a combination of Nonidet P-40 and deoxycholate, presumably due to aggregation, but also induced an altered TAP conformation that was recognized by serum 2p2 but not with mAb 148.3 and 435.3 (not shown) and several other sera reacting with TAP1 and TAP2 transmembrane domain epitopes.

**Effect of Temperature and Nucleotides on the Functional State of TAP Dimers**—Loss of immunoprecipitable TAP complexes expressed in insect cell microsomes after incubation at 37 °C (for human TAP) physiological temperature may be interpreted in several ways. First, the TAP conformation recognized by mAb 148.3 and 435.3 may be typical for non-functional TAP complexes that are intrinsically unstable at 37 °C, an unlikely hypothesis since these mAb precipitate TAP complexes expressed physiologically in human cells. Second, loss of immunoprecipitable TAP complexes may be due to TAP destabilization in insect cell membranes for which 37 °C represents an unphysiologically high temperature. Finally, TAP complexes may normally be stabilized by factors absent from insect cell microsomes, such as HLA class I heavy and/or light chains or tapasin which associate directly with TAP (reviewed in Ref. 26), but also soluble cytosolic compounds, such as energetic nucleotides binding to the ATP-binding cassettes of one or both TAP proteins. To test these hypotheses, peptide binding and transport assays with insect cell and human microsomes were performed.

Preincubation of insect cell microsomes expressing human TAP dimers at 37 °C under the conditions used in immunoprecipitation experiments led to a dramatic decrease of TAP peptide binding capacity, while incubation at 27 °C (the physiological temperature for Si9 insect cells) had no effect on peptide binding (Fig. 2A). A decrease in peptide binding capacity was observed after 1 min of incubation at 37 °C and was essentially complete after 8 min. However, when 2 mM ATP was present during preincubations, loss of peptide binding capacity was strongly inhibited, with 50% of binding capacity (versus less than 3% in the absence of ATP) preserved after 16 min; addition of ATP during preincubation at 27 °C had no effect on subsequent peptide binding. Addition of ATP after preincubation had no effect and did not restore binding capacity, suggesting irreversible loss of binding capacity (not shown). Presence of protease inhibitors during preincubations did not prevent loss of peptide binding, arguing against TAP degradation as the reason for reduced peptide binding (not shown).

Parallel observations were made in peptide transport experiments with insect cell microsomes preincubated at 37 °C (Fig. 2B); peptide transport capacity decreased over time, but was partly preserved by 2 mM ATP present in preincubations. Loss of peptide transport capacity was less dramatic than loss of peptide binding capacity. This may reflect the fact that, due to the strong TAP overexpression in insect cell microsomes relative to the glycosylation machinery, even a relatively small
residual percentage of functionally competent TAP complexes may be sufficient to cause significant accumulation of peptide in the vesicles. In contrast, the level of peptide binding is likely to correspond directly to the number of functionally competent TAP complexes. Leaks of microsomes prepared from baculovirus-infected cells may also contribute to a smaller effect of TAP loss in transport assays.

To test whether the observed effect of incubation at 37 °C was due to the lipid composition of insect cell membranes or to absence of human ER cofactors with a role in antigen processing, analogous peptide transport experiments were carried out with human microsomes (Fig. 2C). TAP-deficient T2 microsomes accumulated only small amounts of glycosylated peptide in the vesicles. In contrast, the level of peptide binding is likely to correspond directly to the number of functionally competent TAP complexes. Leaks of microsomes prepared from baculovirus-infected cells may also contribute to a smaller effect of TAP loss in transport assays.

Effect of Peptide Substrate on the Functional State of TAP Dimers—In view of preservation of TAP function by energetic nucleotides, it was conceivable that peptide substrate might also influence TAP function. An effect of peptide substrates on TAP conformation has been suggested in a different context by a recent study in which peptide was found to inhibit TAP destabilization by the viral inhibitor ICP47 (27). In the experiment shown in Fig. 4, six peptides whose TAP binding affinities had been determined previously (24) and ranged from unmeasurable to high (top to bottom peptides in Fig. 4), were tested for stabilization of functional TAP complexes. Sf9 microsomes expressing TAP complexes were incubated for 5 min with peptides or ATP, then pelleted, resuspended in fresh buffer, and a standard binding assay with peptide R-9-L was performed. Preincubation with peptides at 4 °C affected subsequent binding of [125I]-R-9-L in a fashion that reflected peptide binding affinity; low affinity peptides had no effect, while high affinity binders reduced R-9-L binding up to 50%. This is due to binding competition between labeled R-9-L and residual peptide from preincubation buffer.

After preincubation at 37 °C, a strikingly different result was obtained. Samples preincubated with low affinity peptides (i.e. peptides with 1/IC₅₀ values below 1) bound only 50% of the amount of R-9-L bound after preincubation at 4 °C, and therefore had lost the same amount of functional TAP complexes as samples preincubated at 37 °C without peptide. In contrast, microsomes preincubated at 37 °C with high affinity peptides (1/IC₅₀ greater than 1) bound as much or more peptide R-9-L as samples incubated with the same peptide at 4 °C, suggesting that these peptides maintained TAP complexes in a functional state.

### Footnotes and References

1. TAP1/2-expressing insect cell microsomes were incubated for 12 min at 4 or 37 °C in the presence of the indicated nucleotides (0.5 mM), then washed and used to measure binding of labeled peptide R-9-L. In B, TAP-expressing insect cell microsomes were incubated for 15 min at 4 °C (open symbols) or 37 °C (filled symbols) in the presence of ATP (circles) or ADP (boxes) at the indicated concentration. Then vesicles were pelleted and re-suspended in fresh buffer, and binding of labeled peptide R-9-L was measured.

2. Fig. 3. Efficiency of TAP stabilization by nucleotides. In A, TAP1/2-expressing insect cell microsomes were incubated for 12 min at 4 or 37 °C in the presence of the indicated nucleotides (0.5 mM), then washed and used to measure binding of labeled peptide R-9-L. In B, TAP-expressing insect cell microsomes were incubated for 15 min at 4 °C (open symbols) or 37 °C (filled symbols) in the presence of ATP (circles) or ADP (boxes) at the indicated concentration. Then vesicles were pelleted and re-suspended in fresh buffer, and binding of labeled peptide R-9-L was measured.

3. Fig. 4. Stabilization of TAP by peptide binding. TAP-expressing insect cell microsomes were incubated 5 min at 4 °C (solid bars) or 37 °C (hatched bars) in PBS buffer only (bottom bar), with 2 mM ATP, or in the presence of the peptides (100 μM) with the shown sequences. Peptide affinities shown to the right are expressed as the inverse of previously measured micromolar IC₅₀ values (24); the inverse IC₅₀ of high affinity reporter peptide R-9-L is 5. Following preincubations, vesicles were pelleted, resuspended in cold buffer without ATP or peptides, and a standard binding assay with labeled peptide R-9-L (250 μM) was carried out. To highlight the role of peptide affinity for TAP stabilization, results are expressed as percent. Count/min obtained at 4 °C are expressed as percent of cpm in buffer only (23,031 cpm). Counts/min obtained at 37 °C are expressed as percent of counts/min obtained with the same reagent at 4 °C; for example, microsomes preincubated at 4 °C with peptide ARYQKSTEA bound 65% (14,953 cpm) of reference counts/min, while microsomes preincubated with the same peptide at 37 °C bound 116% of this value (17,249 cpm). N.A., not applicable.

4. TAP proteins (21), and their cytosolic ATP binding cassettes (17, 18), bind di- and trinucleotides and are thought to hydrolyze ATP during peptide transport, although direct evidence for ATP hydrolysis by TAP or its fragments has so far not been obtained. Various nucleotides were therefore tested for their capacity to prevent loss of TAP function. As shown in Fig. 3A, both trinucleotides (ATP, GTP, and CTP) and a dinucleotide (ADP) prevented loss of peptide binding capacity of TAP complexes. A non-hydrolyzable ATP analogue (ATPγS) also prevented loss of TAP function, while addition of AMP or CAMP during preincubations had no effect. In a dose-response experiment (Fig. 3B), both ATP and ADP had no significant effect on binding at 4 °C. At 37 °C, ATP and ADP inhibited loss of TAP function with equal efficiency; an effect was detectable at <10 μM, and preservation of 50% TAP binding capacity required 200 μM nucleotide. These results suggest that a site on TAP or a TAP-associated protein binds di- and trinucleotides (but not mononucleotides) with similar efficiency and stabilizes thereby functional TAP dimers. However, ATP hydrolysis does not appear to be required for preservation of TAP function.

5. In view of preservation of TAP function by energetic nucleotides, it was conceivable that peptide substrate might also influence TAP function. An effect of peptide substrates on TAP conformation has been suggested in a different context by a recent study in which peptide was found to inhibit TAP destabilization by the viral inhibitor ICP47 (27). In the experiment shown in Fig. 4, six peptides whose TAP binding affinities had been determined previously (24) and ranged from unmeasurable to high (top to bottom peptides in Fig. 4), were tested for stabilization of functional TAP complexes. Sf9 microsomes expressing TAP complexes were incubated for 5 min with peptides or ATP, then pelleted, resuspended in fresh buffer, and a standard binding assay with peptide R-9-L was performed. Preincubation with peptides at 4 °C affected subsequent binding of [125I]-R-9-L in a fashion that reflected peptide binding affinity; low affinity peptides had no effect, while high affinity binders reduced R-9-L binding up to 50%. This is due to binding competition between labeled R-9-L and residual peptide from preincubation buffer.

6. After preincubation at 37 °C, a strikingly different result was obtained. Samples preincubated with low affinity peptides (i.e. peptides with 1/IC₅₀ values below 1) bound only 50% of the amount of R-9-L bound after preincubation at 4 °C, and therefore had lost the same amount of functional TAP complexes as samples preincubated at 37 °C without peptide. In contrast, microsomes preincubated at 37 °C with high affinity peptides (1/IC₅₀ greater than 1) bound as much or more peptide R-9-L as samples incubated with the same peptide at 4 °C, suggesting that these peptides maintained TAP complexes in a functional state.
in the presence of ATP and peptide. In or 37 °C in PBS buffer only, or buffer with 2 mM ATP, or 40 somes expressing TAP1 these peptides are given in Fig. 4. AQVPLRPMTYKA (Pep 1) or ARYQKSTEA (Pep 2); TAP affinities of not shown) decreased little or not at all even after a 30-min precipitation followed by Western blots as in Fig. 4, microsomes prepared from human B cell lines T2 (TAP-deficient), 721.221 (HLA-A, -B, and -C deficient), or Jesthom were incubated for 0–30 min at 37 °C in PBS buffer alone or with 2 mM ATP, before TAP immunoprecipitation followed by Western blots as in A. In B, microsomes treated with high affinity binders always had higher peptide binding capacity than untreated microsomes (not shown). However, even peptides with high TAP affinity were significantly less efficient in TAP stabilization than ATP. Effect of ATP and Peptide Substrate on the Conformation of TAP Complexes, Single TAP Subunits, and Cytosolic TAP Fragments—To test whether the effect of ATP and peptide on TAP function was reflected in the recovery of antibody-reactive TAP proteins, insect cell-expressed TAP dimers were incubated in the presence of 2 mM ATP or a peptide with high affinity before immunoprecipitation with mAb 148.3 and analyzed in Western blots stained with the same mAb. In B, microsomes incubation at 37 °C. Two peptides with high TAP affinities shown to preserve TAP peptide binding capacity at 37 °C (Fig. 4) also increased the amount of TAP protein that was precipitated after a 10-min incubation at 37 °C of B cell microsomes, although to a much smaller extent than ATP (Fig. 5C). Thus, results obtained in immunoprecipitations paralleled those of functional experiments, suggesting that dimeric TAP complexes recognized by mAb 148.3 and 435.3 bind and transport peptides and can be stabilized by nucleotides and peptide.

Finally, I wondered whether the effect of temperature on TAP, and its inhibition by nucleotides, were restricted to heteromeric TAP complexes or could also be observed with isolated TAP subunits. Microsomes expressing TAPI or TAP2 only were therefore incubated at 37 °C in the presence or absence of ATP followed by immunoprecipitation with mAb 148.3 and 435.3, respectively. As shown in Fig. 6, incubation at 37 °C affected isolated TAP subunits in the same way as (at least partly) heteromer-assembled TAP proteins. However, while TAPI was efficiently stabilized by ATP, ATP had no effect on the isolated TAP2 subunit. Recombinant proteins corresponding to the predicted cytosolic ABC domains of the two subunits also behaved differently at 37 °C. Recovery of the TAPI ABC domain was not affected by temperature, suggesting that the conformational change of TAPI requires the presence of its transmembrane domains. 37 °C incubation decreased the amount of recoverable TAP2 ABC domain regardless of the presence of ATP; thus, the complete TAP2 subunit and its predicted cytosolic fragment reacted in the same fashion to incubation at 37 °C.

**Figure 5. Stabilization of TAP complexes recognized by mAb 148.3 in the presence of ATP and peptide.** In A, insect cell microsomes expressing TAPI-2 complexes were incubated for 15 min at 4 °C or 37 °C in PBS buffer only, or buffer with 2 mM ATP, or 40 µM high affinity peptide RYQKSTEA; this peptide has an affinity value of 10 (inverse IC50, see Fig. 4). Vesicles were pelleted, lysed in a buffer with 1% Nonidet P-40, and TAP complexes precipitated with mAb 148.3 and analyzed in Western blots stained with the same mAb. In B, microsomes from human B cell lines T2 (TAP-deficient), 721.221 (HLA-A, -B, and -C deficient), or Jesthom were incubated for 0–30 min at 37 °C in PBS buffer alone or with 2 mM ATP, before TAP immunoprecipitation followed by Western blots as in A. In C, TAP immunoprecipitation and blotting was performed by precipitation of Jesthom microsomes for 10 min at 4 or 37 °C in the presence of 2 mM ATP or peptides AQVPLRPMTYKA (Pep 1) or ARYQKSTEA (Pep 2); TAP affinities of these peptides are given in Fig. 4.

**Figure 6. Effect of temperature on isolated TAP subunits and NBD fragments.** Insect cell microsomes expressing TAPI or TAP2 only (left-hand panels), or Nonidet P-40 lysates of insect cells expressing the corresponding ABC fragments (right-hand panels), were incubated for 15 min at 4 or 37 °C in the presence or absence of 2 mM ATP, followed by immunoprecipitation with mAb 148.3 (TAPI, TAP1-ABC) or 435.3 (TAP2, TAP2-ABC) and Western blot analysis using mAb 148.3 or 435.3.
cannot be solubilized in non-ionic detergents, although loss of TAP1 antibody reactivity upon heating after solubilization reported by Russ et al. (21) argues against this hypothesis. It can also not be ruled out that heated TAP complexes associate tightly with other proteins that prevent recognition by mAb 148.3 and 435.3 even under denaturing Western blot conditions.

The most striking phenomenon observed in this study is the stabilization of functional TAP complexes during 37 °C incubations by nucleotides. Although stabilization of an isolated NBD domain of a bacterial ABC transporter has been reported previously (13, 14), this is the first case of an ABC protein requiring nucleotide association for stability. While the known binding of nucleotides to the two TAP NBD domains (17, 18) may appear as a strong argument for TAP stabilization by direct nucleotide binding to the Walker motifs in these domains, other mechanisms cannot easily be ruled out. For example, indirect evidence for nucleotide binding to additional unidentified regulatory sites in P-glycoprotein has recently been reported; however, different from TAP stabilization, ADP was reported to bind more efficiently than ATP to these putative sites (28). Nucleotides may also act on TAP conformation and function via binding to and regulating TAP-associated factors, for example, heat shock proteins. Nevertheless, there is some evidence for a direct implication of the NBD domains and the Walker sequences in TAP stabilization. TAP proteins with certain mutations in the Walker sequences lose peptide binding capacity, demonstrating directly control of TAP function by nucleotide binding to the NBD domains. Moreover, relative efficiencies of various trinucleotides in TAP stabilization correspond to those observed in two studies of nucleotide binding to isolated TAP NBD fragments (17, 18). However, one discrepancy between this report and previous studies of binding of photo-reactive nucleotides to full-length TAP or its NBD domains (17, 18, 21) needs to be mentioned: while ADP inhibits binding of 8-azido-ATP to TAP much less efficiently than ATP, its efficiency in TAP stabilization equals that of ATP. This difference is likely to be related to the fact that transporter labeling by 8-azido-ATP involves nucleotide binding and hydrolysis while TAP stabilization involves nucleotide binding only. As a consequence of its relatively high binding affinity for TAP also demonstrated in previous studies (17, 18, 21), GTP may participate in cellular control of TAP stability.

It is therefore most likely that binding of di- or trinucleotides to the NBD domains stabilizes TAP in 37 °C incubations. Consequently, in the absence of sufficient nucleotide concentrations, incubation at 37 °C would lead to nucleotide dissociation from TAP, either via direct ATP dissociation, or via ATP hydrolysis followed by ADP dissociation. Re-binding of either nucleotide form is sufficient to prevent loss of TAP function, suggesting that ATP hydrolysis is not required to preserve TAP function after nucleotide dissociation. Thus, the findings presented in this study suggest that nucleotide binding to the NBD domains of TAP1, and possibly also TAP2, controls TAP conformation. Conformational changes associated with nucleotide binding affect NBD as well hydrophobic transmembrane domains, as indicated by altered antibody recognition of these domains as well as loss of peptide binding which is thought to involve a binding site formed by several transmembrane domains and/or cytosolic loops between them (25, 29).

Experiments with single TAP subunits and their NBD fragments provide some interesting evidence on the possible mechanisms of TAP destabilization and stabilization. First, the fact that single TAP subunits and the TAP2 NBD are susceptible to the temperature-induced conformational change may indicate that this change is due to ATP dissociation rather than hydrolysis since ATP hydrolysis by ABC proteins from eukaryotic organisms generally requires complete transporter assembly (2). Second, lack of ATP stabilization of isolated TAP2 and its NBD fragment suggests that TAP2 alone may not be capable of re-binding nucleotides after their dissociation (or hydrolysis). Third, ATP stabilization of isolated TAP1 subunits, and of TAP1-assembled (as opposed to isolated) TAP2 subunits suggests that TAP1 alone can re-bind nucleotides after dissociation, and that TAP2 stabilization either involves nucleotide binding to TAP1-assembled TAP2, or is an indirect effect of TAP1 stabilization by nucleotides. Finally, hydrophobic, i.e. membrane-inserted or associated TAP moieties, may initiate the conformational change at 37 °C. This is suggested by the effect of heating on TAP1 and its NBD fragment: recognition of the TAP1 NBD domain by mAb 148.3 (with specificity for its carboxyl terminus) is lost upon heating of complete TAP1, but not of the recombinant NBD fragment.

It needs to be mentioned that Russ and associates (21), who first described evidence for a conformational change in the TAP1 protein during 37 °C incubations, did not observe any effect of nucleotides or peptide. In the case of peptide, this is not surprising since TAP complexes solubilized in strong non-ionic detergents used in the cited study do not bind peptide. The reason for the lack of TAP stabilization by ATP reported by Russ (21) is less evident; it is possible that Triton or Nonidet P-40-solubilized TAP proteins bind ATP with reduced affinity allowing for labeling with photoreactive ATP analogues, but not stabilization of TAP structure. Alternatively, TAP stabilization by nucleotides may involve additional “non-Walker”-binding sites, or third proteins dissociating from TAP during detergent solubilization. In any case, experiments with fully functional microsomal TAP complexes are more likely to reflect physiological conditions than those with detergent-solubilized TAP.

The most surprising result of this study was the clear stabilization of functional TAP complexes by interaction of high affinity peptides with the TAP substrate-binding site. It cannot be ruled out that peptide stabilization of TAP by an indirect mechanism, for example, by increasing TAP affinity for residual ATP or ADP contained in microsome preparations, or by inducing TAP association with an unidentified stabilizing factor. It should be noted that an effect of peptide on TAP structure has been reported recently in a different context: peptides inhibit a conformational change in TAP complexes induced by the viral TAP inhibitor protein ICP47 (27). Although ICP47-incubated resemble heat-treated microsomal TAP complexes with respect to loss of peptide binding and altered conformation (27, 30, 31), the TAP conformations induced by the two treatments must be different since the ICP47 effect is reversible and cannot be inhibited by nucleotides (30, 31).

The observation that microsomes preincubated with high affinity peptides at 4 and 37 °C subsequently bind roughly equivalent amounts of labeled reporter peptide (50% of peptide binding to microsomes preincubated at 4 °C without peptides, see Fig. 4) can be interpreted in several ways. On the one hand, these peptides could maintain all TAP complexes at 37 °C in a functional state, with the reduction of reporter binding by 50% under both conditions resulting from competition with residual unlabeled peptide. In view of the relatively low efficiency of stabilization of antibody-reactive TAP by peptide (Fig. 5), this hypothesis appears unlikely. It seems more likely that high affinity binders stabilize only a rapidly diminishing fraction of TAP complexes at 37 °C (as seen in immunoprecipitation ex-

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2 S. Daniel and P. M. van Endert, manuscript in preparation.

3 P. M. van Endert, unpublished data.
experiments); however, these TAP complexes may be more readily available for binding of reporter peptide than complexes preincubated with the same peptide at 4 °C. Such higher TAP availability could, for example, be due to peptide degradation during preincubations at 37 °C. Moreover, TAP complexes preincubated at 4 °C may retain large amounts of unlabeled competitor peptide by binding it. In contrast, TAP complexes preincubated at 37 °C may rather be stabilized by transitory interaction with peptide during transport and release peptide which can then be eliminated by degradation or subsequent washing. Again, this would lead to lower concentrations of unlabeled competitor peptide after 37 °C preincubations. The latter model also implies a slow transition of TAP to the nonfunctional state after ATP and peptide release, a notion that is compatible with the kinetics of loss of TAP function observed in this study.

What is the potential physiological significance of TAP stabilization by nucleotides? On the one hand, it is tempting to speculate that the state assumed after 37 °C incubation is related (although evidently not identical) to a step occurring during physiological peptide transport. Current models of substrate transport by ABC proteins assume a transitory “nucleotide-off” state (32). It can be speculated that, in the case of TAP, this normally transitory state may be highly unstable and prone to undergo irreversible conformational changes unless rapidly reversed by nucleotide binding. Lack of peptide binding to TAP complexes in the putative nucleotide-off conformation would then be consistent with a role of this state in peptide release by the transporter. Another potential physiological role for TAP stabilization by nucleotides may be to limit HLA class I-restricted antigen presentation by stressed cells. In this scenario, stress-associated decrease in the cytosolic level of energetic nucleotides would reduce the number of functional TAP complexes and thereby peptides supplied to HLA class I molecules. Such a mechanism may, for example, serve to contain the risk of autoimmunity directed against protein breakdown products presented by stressed cells. Tight control of transporter function by cellular nucleotide levels has also been suggested for the cystic fibrosis transmembrane conductance regulator, another ABC protein (33).

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