Functional Role of C-Terminal Sequence Elements in the Transporter Associated with Antigen Processing

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TAP delivers antigenic peptides into the endoplasmic reticulum (ER) that are subsequently bound by MHC class I molecules. TAP consists of two subunits (TAP1 and TAP2), each with a transmembrane (TMD) and a nucleotide-binding (NBD) domain. The two TAP-NBDs have distinct biochemical properties and control different steps during the peptide translocation process. We noted previously that the nonhomologous C-terminal tails of rat TAP1 and TAP2 determine the distinct functions of TAP-NBD1 and -NBD2. To identify the sequence elements responsible for the asymmetrical NBD function, we constructed chimeric rat TAP variants in which we systematically exchanged sequence regions of different length between the two TAP-NBDs. Our fine-mapping studies demonstrate that a nonhomologous region containing the \( \alpha6/\beta10 \)-loop in conjunction with the downstream switch region is directly responsible for the functional separation of the TAP-NBDs. The \( \alpha6/\beta10 \)-loop determines the nonsynonymous nucleotide binding of NBD1 and NBD2, whereas the switch region seems to play a critical role in regulating the functional cross-talk between the structural domains of TAP. Based on our findings, we postulate that these two sequence elements build a minimal functional unit that controls the asymmetry of the two TAP-NBDs.

region appear to be critical for proper transduction of conformational signals between the structural domains of TAP.

This is the first report in which the functions of the α6/β10-loops and the switch region of TAP have been structurally defined. Our experimental findings provide new insights into the sequence requirements for the functional divergence of the TAP-NBDs and suggest that molecular regulatory mechanisms in TAP differ from the postulated C-terminal regulation of the ABC-protein SUR (16).

Materials and Methods

Cell lines and cell culture

T2 is a human lymphoblastoid cell line that lacks both TAP genes and expresses only the HLA-A2 and -B5 class I molecules (19). Transfectants of T2 cells containing wild-type rat TAP* (20) and TAP chimeras were cultured in IMDM (Invitrogen Life Technologies) supplemented with 10% of T2 cells containing wild-type rat TAPα (20) and TAP chimeras were transfected into T2 cells by electroporation using a gene pulse (Bio-Rad) at 270 V and 500 μF. After selection with G418 (1 mg/ml) for 4–6 wk, stable transfectants were subcloned and screened for TAP chain expression by Western blotting.

Antibodies

116/5 is a polyclonal rabbit antiserum specific for the C terminus of rat TAP2 chains (20). D90 is a polyclonal rabbit antiserum recognizing the C terminus of rat TAP1 chains (23). MAC 594 is a monoclonal mouse anti-rat TAP2 Ab (24) derived from immunization with the recombinant histidine-tagged cytoplasmic domain of rat TAP2. MAC 594 recognizes the polypeptide residues at positions 538 and 539 in the core NBD of rat TAP2* (11). 1p3 is a polyclonal rabbit antisera that binds epitopes in or between the predicted TMDs of TAP1 (25). 4E is a conformation-dependent mAb that recognizes an epitope common to all HLA-B and -C Ags (26).

Immunoprecipitation, Western blotting, and flow cytometry

For immunosolubilization experiments, 1×10⁶ cells were washed twice in ice-cold PBS (1.7 mM K₂HPO₄, 10 mM Na₂HPO₄, 140 mM NaCl, and 2.7 mM KCl, pH 7.5) before solubilization in lysis buffer (PBS [pH 7.5] containing 1% Triton X-100). Immunoprecipitations with anti-TAP (116/5) and anti-TAP1 (1p3) were performed as described previously (27).

Antibodies were visualized with HRP-conjugated secondary Abs (donkey anti-rabbit IgG-HRP; Amersham Biosciences) and ECL substrate (Amersham Biosciences). Flow cytometry experiments using the anti-HLA-B5-specific mAb 4E were performed as described previously (27).

Transport assay, peptide cross-linking, and nucleotide-binding assays

For transport assays, 2×10⁶ cells were permeabilized with streptolysin O (2 U/ml; Murex). After washing with PBS, 0.5 μM radiolabeled peptide S₈ (TVDNKTRYR, in the single-letter amino acid code), 10 mM ATP, and incubation buffer (50 mM HEPS, pH 7.5, 250 mM sucrose, 150 mM CH₃COOK, 5 mM CH₃COO-Mg₂⁺, 10 mM CH₃COO-H₂O, 1 mM DTT, and 1 mM Pefabloc (Roche), and 1.8 μg/ml aprotinin (Sigma-Aldrich)) were added and incubated for 10 min at 37°C. After lysis with 20 mM Tris-HCl (pH 7.5), 500 mM NaCl, and 0.1% Nonidet P-40 (Sigma-Aldrich), transport-glycosylated peptides were isolated with Con A-Sepharose (BD Biosciences) and quantitated by gamma counting (28). For peptide cross-linking, permeabilized cells were incubated with 1 μM radiolabeled and N-hydroxysuccinimide-activated 8-azido-ATP (H₈-ATP) and 8-azido-ADP-agarose (Sigma-Aldrich). To analyze peptide-induced ATP binding, CHAPS-solubilized cell membranes were pretreated with increasing concentrations of S₈ peptide (10, 1, 0.1, and 0.01 μM) for 30 min. Afterward, lysates were incubated with ATPagarose for 30 min. After washing, the bound proteins were eluted with SDS loading buffer.

For photolabeling of TAP with radiolabeled 8-azido-ATP, membranes of cells were prepared and resuspended in 250 mM sucrose, 50 mM KCl,
2 mM MgCl₂, 2 mM EGTA, and 10 mM Tris-HCl (pH 6.8) (27). Membranes corresponding to 3 × 10⁶ cells in a final volume of 100 μl were incubated with 2 μM 8-azido-[α-³²P]ATP (ICN Biomedicals) for 5 min at 4°C. Cross-linking was induced by irradiation with a UV lamp at 254 nm for 5 min at 4°C.

Preparation of microsomal membranes

Microsomes from 1 × 10⁸ T2 cells expressing chimeric and wild-type TAP proteins were generated by sucrose gradient fractionation (29). Cells were counted twice with ice-cold PBS, resuspended in 10 ml of 10 mM Tris-HCl (pH 7.5) with protease inhibitor mixture (Complete Protease Inhibitor; Roche), and incubated on ice for 10 min. The lysed cells were then homogenized and centrifuged at 800 × g for 5 min at 4°C. The resulting supernatants were resuspended in 5 ml of 1.3 M sucrose buffer (20 mM HEPES (pH 7.5), 25 mM CH₃COOK, 5 mM (CH₃COO)₂Mg · 4H₂O, 1 mM DTT, and protease inhibitor mix) and centrifuged again at 800 × g at 4°C for 10 min. The supernatants were then centrifuged at 68,000 × g at 4°C for 2 h, and the membrane pellets were resuspended in 800 μl of 0.25 M sucrose buffer. Subsequently, 5.6 ml of 2.5 M sucrose gradient buffer was added, and the suspension was overlaid carefully with 2.9 ml of 2 M sucrose buffer. Subsequently, 5.6 ml of 2.5 M sucrose gradient buffer was added, and the suspension was overlaid carefully with 2.9 ml of 2 M sucrose buffer. Approximately 800 μl of 0.25 M sucrose buffer was carefully loaded on the top of the gradient. The sucrose gradient was centrifuged at 100,000 × g for 1 h at 4°C. The microsomes were collected at the interface between the 2 and 1.3 M sucrose buffer, diluted in 20 ml of HEPES buffer (20 mM HEPES (pH 7.5), 25 mM CH₃COOK, 5 mM (CH₃COO)₂Mg · 4H₂O, 1 mM DTT, and protease inhibitor mixture), homogenized, and centrifuged at 68,000 × g at 4°C for 1 h. The microsomal pellets were resuspended in 200 μl of 20 mM HEPES buffer. Finally, aliquots of 30–50 μl were snap-frozen in liquid nitrogen and stored at −80°C.

Chemical cross-linking

Membranes from 2 × 10⁷ T2 cells were resuspended in 100 μl of PBS containing 1% digitonin at 4°C, and the homobifunctional cross-linker ethylene glycol bis succinimidyl succinate (EGS; Pierce) was added to a final concentration of 3 mM. The primary targets for EGS are ε-amino groups of solvent-exposed lysine residues. After incubation for 30 min at 4°C, the lysed membranes were washed with PBS containing 1% digitonin. The nonameric TAP-binding peptide S8 was added to a final concentration of 15 μM for 1 h at 4°C before EGS cross-linking.

Computational homology modeling of the NBD of rat TAP2

Model building was performed by a conventional three-step procedure. Step 1 was alignment. A global multialignment was obtained with the help of vector NTI using a modified version of CLUSTAL V (30). The sequence of human TAP1 (accession no. Q05318) was read directly from the crystal structure (18). Sequence homologies among human TAP1 and rat TAP2 (accession no. X63854) are in the range of 70%. Secondary structure predictions of the rat TAP-NBD2 sequence were performed with PHD (31) and PSIPRED v2.3 (32). Step 2 was model building. An initial model of the rat TAP-NBD2 main chain was built by searching a database of loops obtained from highly resolved protein structures (33). Loop selections were made on the basis of minimal steric interactions with the rest of the model. A full atom model of the rat TAP-NBD2 ectodomain was generated with the help of the backbone-dependent rotamer library implemented in SC-WRL 3.0 (34). Step 3 was refinement of the structure model. Refinement of the rat TAP2-models was performed using the GROMACS package (35). The solvated rat TAP-NBD2-models were energy-minimized until they converged using the steepest descent method. A molecular dynamics-simulated annealing simulation (simulation time, 300 ps; temperature, 0–300 K), followed by a second energy minimization, were used to optimize the conformation of the newly introduced loops. During simulated annealing, positional constraints were applied to all main chain atoms, except atoms of newly introduced loops.

Results

Chimeric studies on the C-terminal regulation of asymmetrical nucleotide binding and NBD function in TAP

We have previously shown that the distinctive nucleotide-binding behavior of NBD1 and NBD2 in the resting state of rat TAP (9, 10) depends directly on the divergent C-terminal tails, rather than on the core NBDs situated between the conserved Walker A and B motifs (11). A chimeric rat TAP2 chain, containing the core NBD2 fused to the C-terminal segment of rat TAP1, adopts the ATP-binding behavior of wild-type NBD1, whereas a corresponding rat TAP1 chimera, containing the core NBD1 fused to the C-terminal segment of rat TAP2, shows the characteristic ADP-binding properties of wild-type NBD2 (11). Moreover, a rat TAP chain chimera, designated 1C2, comprising TMD1 (residues 1–505) and the core NBD2 (residues 494–639) with the C terminus of TAP1 (residues 653–725), acquires not only the nucleotide-binding behavior, but also the functional properties of TAP1 (11). Thus, the TAP chain chimera 1C2 provides a suitable experimental system for fine-mapping the regions within the C terminus that are responsible for the distinct nucleotide-binding properties of the TAP-NBDs.

We constructed a series of chimeric rat TAP chains based upon the variant 1C2 in which we dissected the nonhomologous C-terminal tail into four portions by progressively exchanging sequences between the two TAP-NBDs (Fig. 1). Positions of secondary structure elements in the C-terminal tails of rat TAP1 and rat TAP2 (see Fig. 1) were predicted on the basis of the crystal structure of the human TAP-NBD1 (18). The designations ID2, 1E2, 1F2, and 1G2 indicate four different chimeric TAP chains where NBD1 regions between residues 506–659, 506–670, 506–689, and 506–705 were replaced by the corresponding NBD2 sequence: residues 494–646, 494–653, 494–672, and 494–685, respectively (Fig. 1). Because recent findings have suggested that the β11/β12 region plays an important role in determining the nucleotide-binding properties of the ABC protein SUR (16), we created an additional rat TAP chain variant (1H2) from the chimera 1C2 in which the β11/β12 sequence of TAP2 (residues 673–685) was replaced by the corresponding sequence segment of TAP1 (residues 690–705; Fig. 1). All the constructs contained TMD1 as well as core NBD2 and only differed in their C-terminal sequences. After stable transfection of the different TAP chimeras into the TAP-negative human cell line T2, we tested the steady state expression of each TAP chain by immunoblot analysis using different TAP-specific Abs (see Materials and Methods). As shown in Fig. 2A, all chimeric TAP chains were stably expressed at similar levels.

To investigate whether the progressive exchange of sequence between the two TAP-NBDs has an effect on the nucleotide-binding properties of the chimeric TAP chains, lysates of ER membrane preparations were analyzed by standard nucleotide-binding assays (6, 24) for binding to ATP- and ADP-agarose beads (Fig. 2B). The binding to the different nucleotide-agaroses was compared with that of wild-type TAP subunits, the chimera 1C2 (11), and chimera 1N2, which comprises TMD1 and the entire sequence of NBD2 (9) (Fig. 1). Analysis of TAP1, TAP2, 1C2, and 1N2 (Fig. 2B, left panel) confirmed that the distinct nucleotide binding of the TAP subunits is an inherent property of the NBDs that is determined by the C-terminal tails (11). The variant 1C2 bound in a TAP1-like manner to ATP- as well as ADP-agarose (11), whereas 1N2 interacted only with ADP-agarose, which is a characteristic property of TAP2 (5). Analysis of the other chimeric TAP chains showed that 1D2 bound to ATP- as well as ADP-agarose, whereas the chimical polypeptides 1E2, 1F2, 1G2, and 1H2 bound only to ADP-agarose (Fig. 2B, right panel).

To explore whether the TAP chain variants can participate as a TAP1-type chain in a functional heterodimeric peptide transporter, we created five different TAP molecules in T2 cells by coexpressing the chimeric variants 1D2, 1E2, 1F2, 1G2, and 1H2 in combination with wild-type TAP2. The expression levels of these TAP variants were comparable to those of wild-type transporters (Fig. 3A, left panel) and showed normal subunit assembly (Fig. 3A, right panel, upper part).

For functional characterization, the different transporter variants were first analyzed for their peptide- and ATP-binding behaviors.
Binding of peptide and nucleotide was analyzed by photolabeling experiments using radioactively labeled and UV-cross-linkable reagents (5). Although all chimeric transporter variants had the capacity to bind peptides, only the variant TAP2-1D2 showed a pattern of ATP binding comparable to that of the functional wild-type transporter (5). In line with the nucleotide-binding assays shown in Fig. 2B, the ATP cross-linking experiments showed detectable ATP labeling for TAP1 in the wild-type transporter and for 1D2 in the chimeric transporter TAP2-1D2, whereas all other transporter variants showed no detectable ATP cross-linking. We measured the peptide transport activities of the different chimeric transporters in standard transport assays (28) using radioiodinated model peptides (5). As shown in Fig. 3B, only TAP2-1D2 showed a significant transport activity compared with wild-type TAP and the transporter variant 2-1C2. The transport efficiency of TAP2-1D2 was ~40–50% that of wild-type TAP and ~70% that of TAP2-1C2. Thus, like the chimera 1C2 (11), the chimeric polypeptide 1D2 can functionally substitute for wild-type TAP1, albeit inefficiently. In contrast, no detectable peptide transport above that observed for the nontransfected T2 cell line was seen for variants TAP2-1E2, TAP2-1F2, TAP2-1G2, and TAP2-1H2 (Fig. 3B). The different peptide transport activities of the chimeric TAP molecules were also reflected in the surface expression of mature HLA-B5 molecules determined by flow cytometry (Fig. 3C).

Taken together, our data show that the C-terminal sequence information from TAP1 present in the chimera 1D2 confers the nucleotide-binding and functional characteristics of NBD1 upon NBD2, whereas the C-terminal amino acids of NBD1 present in the chimeras 1E2, 1F2, 1G2, and 1H2 do not support an NBD1-like function. Thus, in contrast to the ABC protein SUR (16), our findings clearly demonstrate that in the case of TAP, a region upstream of the β9β10-loop and switch region build a minimal functional unit that controls asymmetry of the two TAP-NBDs.

The differences in nucleotide-binding and peptide transport functions of 1D2 and 1E2 (Figs. 2 and 3) suggest that the sequence stretch between the secondary structure elements α6 and β10 (α6/β10-loop, residues 660–670 in rat TAP1, and residues 647–653 in rat TAP2; see Fig. 1) could play a crucial role in controlling the biochemical and functional behaviors of the TAP-NBDs. Sequence differences in nucleotide binding and peptide transport. The Journal of Immunology.
alignment of rat TAP-NBD1 and rat TAP-NBD2 (Fig. 1) shows that this NBD segment is strikingly different in sequence (RLLY-ESPEWAS vs TWRSQED, in the single-letter amino acid code) and in length (11 residues vs seven residues) between rat TAP1 and TAP2. The same is also true for the TAP-NBDs of other species, e.g., mouse, hamster, and human. Three dimensional modeling of the rat TAP-NBD2 primary sequence using the NBD1 of human TAP (18) as a structural template (Fig. 4A) suggests that the αβ/β10-loop of TAP2 is also structurally divergent (RMS deviation, 5.5 Å) from that found in TAP1.

To investigate whether the nature of the αβ/β10-loop controls the distinctive nucleotide-binding behavior of the TAP chains, we constructed a 1D2-related chimera (1L2) in which we exchanged only the sequence of the αβ/β10-loop between NBD1 and NBD2 (residues 660–670 in NBD1, residues 647–653 in NBD2; Fig. 4B). In addition, we created a chimeric construct (1S2) in which the sequence exchanged between NBD1 and NBD2 extended from the αβ/β10-loop to include the nonhomologous residues of the downstream switch region (residues 660–689 in TAP1, residues 647–672 in TAP2; Fig. 4B). The switch region is thought to play a crucial role in the conformational signal transduction of ABC transporter domains (36). After stable transfection of both chimeric TAP constructs into T2 cells (Fig. 4C, left panel), we compared the nucleotide-binding capacities of 1L2 and 1S2 with those of the wild-type TAP chains by performing nucleotide-binding assays with ATP- and ADP-agaroses. As can be seen from the analysis in Fig. 4C (right panel), both chimeras 1L2 and 1S2 appear to have the same ATP- and ADP-binding behavior as wild-type TAP1, indicating that the sequence information of the αβ/β10-loop of TAP1 alone has the capacity to confer NBD1-like nucleotide-binding properties upon NBD2.

To analyze whether the chimeric TAP chains 1L2 and 1S2 acquire the functional properties of wild-type TAP1, we created two TAP variants in T2 cells by coexpressing 1L2 or 1S2 with wild-type TAP2. Expression levels of two variants, TAP2-1L2 and TAP2-1S2, were comparable to those of wild-type TAP (Fig. 5A, right panel) and showed a balanced subunit assembly (Fig. 5B, middle part). Photo-cross-linking of peptide substrates and 8-azido-ATP was performed on membrane preparations of the T2 transfectants and assessed for labeling of TAP polypeptides as described above (see Fig. 3). For the resting states of both chimeric transporters TAP2-1L2 and TAP2-1S2, we observed peptide- (Fig. 5A, right panel, middle part) and ATP-binding properties (Fig. 5A, right panel, bottom part) indistinguishable from those of the functional wild-type transporter molecules. Furthermore, peptide transport assays (Fig. 5B, left panel) revealed that peptide translocation activity was clearly detectable for TAP2-1S2 (35–40% of wild-type TAP), consistent with the elevated HLA-B5 surface expression data for the corresponding T2 transfectant (Fig. 5B, right panel). In contrast, however, the variant TAP2-1L2 was completely transport-inactive (Fig. 5B, left and right panels). In view of the different biochemical and functional behaviors of 1D2 and 1E2 (Figs. 2 and 3), our experiments on TAP2-1L2 (Fig. 5) suggest...
FIGURE 3. Functional properties of chimeric transporters 2-1D2, 2-1E2, 2-1F2, 2-1G2, and 2-1H2. A, Expression levels of wild-type and chimeric transporters (left panel). Cells were lysed in 1% Triton X-100. Lysates of 5 × 10^4 cells were separated by 7.5% SDS-PAGE and blotted onto nitrocellulose membranes. Immunoblots were probed for TAP chains using antisera D90 (C-term. NBD1) and 116/5 (C-term. NBD2). Complex formation of wild-type and chimeric TAP transporters is shown (right panel, top). Transfected T2 cells were lysed in 1% Triton X-100, and TAP molecules were immunoprecipitated with anti-TAP2 antisera 116/5 (TAPwt, TAP2-1D2, TAP2-1E2, TAP2-1F2, and TAP2-1G2) or 1p3 (TAP2-1H2). After washing, the bound proteins were eluted with SDS, separated on a 7.5% SDS-gel, and analyzed by probing Western blots for TAP-NBD1 (D90) or TAP-NBD2 (116/5). Peptide-binding properties are shown (right panel, middle). The peptide-binding activity of the TAP variants was analyzed by substrate cross-linking. Microsomal fractions were resuspended in binding buffer and incubated with 1 μM radioiodinated and HSAB-conjugated peptide S8. After cross-linking, membranes were lysed, and TAP was immunoprecipitated with anti-TAP2 antiserum (116/5). The nucleotide-binding properties of chimeric TAP molecules are shown (right panel, bottom). Membrane fractions of T2 cells expressing wild-type or chimeric transporters were lysed in lysis buffer containing 2 μM radiolabeled 8-azido-ATP. After UV cross-linking, TAP variants were immunoprecipitated with an anti-TAP2 antiserum and separated by SDS-PAGE. B, TAP-mediated peptide transport. Transfected and nontransfected T2 cells were permeabilized with streptolysin O (SLO) and incubated in transport buffer containing 10 mM ATP and radioiodinated peptide S8 for 10 min at 37°C. Bar graphs show the recovered amount of transported labeled peptides as cpm and represent the average values of experiments performed in duplicate. C. Surface expression of MHC class I molecules. Cells were incubated with mAb 4E that recognizes HLA-B5, followed by FITC-labeled secondary Ab. Surface expression of HLA-B5 was detected by flow cytometry ((panel)). Mean fluorescence intensity values are indicated. Background staining was determined by incubating only with secondary Ab (□). TAP variants are shown schematically.
that the α6/β10-loop of TAP1 is necessary, but clearly not sufficient, to confer TAP-NBD1-like function upon TAP-NBD2. Furthermore, the functional phenotype of the chimera 1S2 (Fig. 5) and the nonfunctional phenotype of the chimera 1E2 (Figs. 2 and 3) indicate that the switch region of TAP-NBD1 alone is also not sufficient to alter the functional character of NBD2.

We conclude that the nonconserved α6/β10-loop and the switch region build a minimal functional unit that controls different aspects of the distinctive behavior of the TAP-NBDs. In the resting state of TAP, sequence differences in the α6/β10-loop determine the nonsynonymous nucleotide binding of NBD1 and NBD2, whereas the two different switch regions are probably required to initiate the translocation process upon nucleotide and peptide binding.

Asymmetrical sequence character of the switch region is critical for the functional cross-talk between the TAP domains

In line with our data presented above (Figs. 4 and 5), studies of different ABC transporters have suggested that the switch region is not implicated in nucleotide binding, but could play an important role in transducing information about different conformational states between the structural domains (36). In the case of TAP, it has been demonstrated that peptide binding has an allosteric effect on the ATP-binding capacity (6) and stimulates ATPase activity in a functional peptide transporter (37). The differences in the transport activity of TAP2-1L2 and TAP2-1S2 (Fig. 5) suggest that the nonhomologous sequences of the switch regions in TAP1 and TAP2 might be important for the correct functional interplay.
between the TAP domains upon peptide binding. To investigate this, we first compared the intrinsic ATP-binding capacities of the chimeras TAP2-1L2 and TAP2-1S2 with that of the wild-type transporter by affinity chromatography using ATP-agarose and elution of bound polypeptides with increasing concentrations of free MgATP (0–10 mM). As shown in Fig. 6A, both chimeric transporters were as efficiently eluted by MgATP as was the wild-type transporter, with a 70% release at 1 mM MgATP. This shows that...
both chimeric transporter variants have intrinsic ATP affinities similar to that of wild-type TAP. Based on the observation by Karttunen et al. (6) that peptide binding to wild-type TAP stimulates the ATP-binding capacity of the NBDs, we compared the ATP-binding behaviors of wild-type and the chimeric transporters by nucleotide affinity chromatography in the presence of increasing concentrations of peptide substrate. In confirmation of previous findings (6), the wild-type transporter showed a clear peptide-induced ATP binding with a 2- to 3-fold increase at 10 μM S8 peptide (TVDNKTRYR, in the single-letter amino acid code). A similar increase in ATP-binding behavior could be observed for the transport active variant TAP2-1S2. In contrast, however, in the case of the inactive variant TAP2-1L2, no peptide-induced ATP binding was observed (Fig. 6B), suggesting that the block of peptide transport of TAP2-1L2 is due to a defect in the functional interplay between peptide- and nucleotide-binding sites.

**Discussion**

The two NBDs of the antigenic peptide transporter TAP have different nucleotide-binding properties and seem to control different steps in the ATP-driven peptide transport cycle (4–6, 8). Several findings suggest that the catalytic function of TAP-NBD2 controls the peptide-binding properties of TAP, whereas ATP-hydrolysis in TAP-NBD1 accompanies the translocation of peptide from the cytosol into the lumen of the ER (6, 8, 9). Our previous studies have demonstrated that the distinct nucleotide-binding properties and functions of the TAP-NBDs are mainly determined by the nonhomologous C-terminal tails of the two TAP chains (11).

To gain insight into how the C-terminal tails regulate the activity of antigenic peptide transporter TAP, we have searched for discrete sequence elements that determine the distinct biochemical behaviors of NBD1 and NBD2. We approached this problem by
systematically exchanging the sequences of TAP1 and TAP2 to create different chimeric TAP chains (Figs. 1 and 4A). We have been able to identify two sequence elements in the nonhomologous C-terminal tails, namely the α6/β10-loop and the switch region, that are of critical importance for the distinct functions of the two TAP-NBDs.

The analysis of the chimeric TAP chains ID2, IE2, IF2, and IG2 (Figs. 2 and 3) and the results obtained for chimeras IL2 and IS2 (Figs. 4 and 5) clearly demonstrate that the nonhomologous α6/β10-loop is the crucial determinant that controls the distinct ATP-binding behaviors of the two TAP-NBDs. Structural analysis of various ABC transporters and their NBDs (18, 40 – 42) showed that the α6/β10-loop lies adjacent to the switch region and is in close proximity to the Walker B motif (18, 40, 41). It has been previously shown for the TAP-related P-glycoprotein that a region in NBD1 containing the sequence between α6 and β10 cannot be substituted by the corresponding sequence stretch of NBD2 without affecting transport function (43). Thus, this NBD region seems to be important for molecular processes that determine the functional separation of the two NBDs of P-glycoprotein (44, 45). Furthermore, residues in the α6/β10-loop region also seem to be involved in controlling the proper NBD function of bacterial ABC transporters (46). However, there is no indication from structural or biochemical studies that the α6/β10-loop region makes direct contact with bound ATP and/or ADP (18, 40 – 42). Thus, it is tempting to speculate that the conformational constraints imposed by the different α6/β10-loops might affect the access of nucleotides to the TAP-NBDs. The α6/β10-loops could participate in structural deviations of the two TAP-NBDs that are critical for the different nucleotide-binding capacities of TAP-NBD1 and -NBD2. In support of this idea, the biochemical studies of several groups have suggested that NBD1 and NBD2 of TAP have similar nucleotide-binding affinities, but differ in their ATP accessibility (6, 47).

As mentioned above, a characteristic feature of the α6/β10-loop regions in TAP-NBD1 and -NBD2 is their striking difference in sequence and length (Fig. 1). This is not only true for the rat TAP subunits, but also for the TAP chains of other species. Although caution is required when assessing the significance of three-dimensional structure modeling, our data for TAP-NBD2 (Fig. 4A), based on the crystal structure of the human TAP-NBD1 (18), indicate that the α6/β10-loop may be one of the most significant structural differences between the two TAP-NBDs. This could reflect a structural adaptation to the requirements of nonconsynonymous ATP binding in TAP1 and TAP2. Most interestingly, Karttunen et al. (6) have provided experimental evidence that in the resting state of TAP, the conformation of TAP-NBD2 is in a structurally more closed state, whereas the nucleotide-binding site of TAP1 seems to be open and solvent-accessible. In line with this, preliminary studies by our own group indicate that the α6/β10-loop contributes to the regulation of nucleotide binding in a conformational, rather than a sequence-specific, manner (R. M. Leonhardt and M. R. Knittler, unpublished observation).

Crystallization of TMDs of prokaryotic ABC transporters has revealed that the sequence between α6 and β10 connects two structurally mobile regions, the D-loop and the switch region, that seem to control NBD conformation in a dynamic and nucleotide-dependent manner (42). To elucidate the structure/function relationship of the α6/β10-loops in TAP, information on the tertiary structure of both TAP-NBDs will be essential. Attempts to purify and solve the structure of TAP-NBD2 are being conducted by our laboratory.

Recent studies on the ABC protein SUR have identified a sequence in the β11/β12 region of the C-terminal tails that determines the different nucleotide-binding and functional properties of that protein and its subtypes (16). It is thought that the functional role of the NBDs of the ABC protein SUR differs from that of ABC transporters such as TAP, which, in contrast to SUR, use nucleotides to pump substrates across cellular membranes (48). In line with this idea, our present findings suggest that the regulation of NBD function by the C terminus of SUR and TAP is based on a different molecular mechanism. The studies shown in Figs. 2B and 3A indicate that sequence differences in the β11/β12 regions of the TAP chains make no contribution to the distinctive nucleotide-binding behaviors of the TAP-NBDs.

The crucial finding of our work on the TAP chain chimeras IL2 and IS2 (Figs. 4 and 5) is that the functional separation of the TAP-NBDs is essentially controlled by two adjacent sequence elements: the α6/β10-loop and the switch region. Our experiments shown in Figs. 5 and 6 suggest that sequence differences in the switch region are not responsible for the distinct nucleotide-binding behaviors of TAP1 and TAP2 (Fig. 5), but seem to be required for the correct transfer of conformational signals between peptide-binding site and NBDs (Fig. 6). Karttunen et al. (6) have previously demonstrated that peptide binding to TAP induces conformational changes in the NBDs, favoring the binding of ATP. Similar observations of substrate-enhanced ATP binding have been made for other ABC transporters (49, 50). The functional TAP variant 2-1S2 shows the same peptide-enhanced ATP binding as wild-type TAP, whereas almost no peptide effect was observed for TAP2-1L2 (Fig. 6B), containing two identical NBD2 switch regions. A comparison of the TAP variants 2-1L2 and 2-1S2 (Fig. 6) indicates that peptide-induced ATP binding depends on the functional behavior of the TAPI-like subunit. This supports the hypothesis that ATP binding and hydrolysis by TAP-NBD1 accompany the transport cycle after peptide binding to TAP (4, 11). Because TAP2-1L2 can bind peptide substrates (Fig. 5A) and has normal ATP-binding behavior in the absence of additional substrates (Fig. 6A), it is likely to be folded correctly. It is reasonable to assume that the defect in the peptide-enhanced ATP binding in TAP2-1L2 results from a block in the conformational signaling between the structural domains of TAP. In support of this view, we could show that peptide binding to wild-type TAP and TAP2-1S2, but not to the variant TAP2-1L2, brings solvent-exposed portions of the TAP domains together and facilitates EG cross-linking of the subunits (Fig. 6C). Although the exact sites of EG cross-linking in TAP remain to be determined, our findings presented in Fig. 6 suggest that the presence of distinct switch regions of TAP1 and TAP2 is indispensable for the normal functional interplay of TAP domains and subunits.

It is well established that substrate binding by TMDs of ABC transporters triggers conformational rearrangements in the NBDs (51). However, the mechanisms of interdomain communication are still speculative. Unfortunately, because none of the present ABC transporter structures (52, 53) was determined with a bound substrate, they provide only limited information on the conformational interplay between substrate and nucleotide-binding sites. However, the existing data for bacterial ABC transporters indicate that a reorientation of the NBDs is required to stabilize the binding of ATP (51). The mutual conformational cross-talk between the TMDs and the ATP-binding sites seems to be mediated and controlled by the α-helical subdomains of the NBDs (54), which contain the conserved and conformationally mobile Q-loop. Recent findings provide evidence that the structural changes in the α-helical subdomain are coupled to a repositioning of sequences (42) that are probably involved in the formation of the NBD dimer interface (53). These sequence elements include the D loop and the switch region. Thus, the enhancement of ATP binding upon peptide binding could result from stabilization of the TAP structure in a particular conformation or, more specifically, could be due to
altered NBD-NBD interactions that enhance nucleotide binding. In view of the phenotype of TAP2-IL2, it is interesting to note that mutations in the switch region of the bacterial ABC transporter HisQMP2 affect not only the catalytic activity, but also the normal interdomain communication between paired NBDs (55).

It is notable that TAP2 contains the consensus sequence of the switch region, whereas TAP1 has a glutamine in place of the conserved histidine found in most pro- and eukaryotic ABC transporters. However, despite our experimental efforts, to date we have been unable to identify the discrete amino acid residues responsible for the functional nonequivalence of the switch regions in rat TAP1 and rat TAP2, suggesting that the asymmetrical character of the switch regions might not be confined to a single nonhomologous residue. Clearly, additional studies are needed to characterize the precise role(s) of the switch region in the interdomain signaling of the two TAP chains.

Our previous (11) and present experiments (Figs. 4–6) demonstrate that sequence differences within the core NBDs are not essential for the functional asymmetry of the TAP-NBDs. However, this does not exclude the possibility that differences between the sequence elements of the ATP-binding cassettes could contribute to some extent in the nonsynonymous functions of TAP1 and TAP2. A clear and important finding of our present work is that functional separation of TAP-NBD1 and TAP-NBD2 depends on more than one distinct sequence element in the NBDs. Recent experiments on the TAP-related tandem ABC transporter multidrug-resistant protein 1 (MRP1) (56) suggest that sequence differences at the C-terminal end of the Walker B motifs (aspartate or glutamate, respectively, which are also present in TAP1 (LILLID) and TAP2 (LILLE), contribute to the functional divergence of the NBDs. In this context it should be noted that MRP1 (49, 57) is not homozygous deletions that simultaneously eliminate expressions of class I and class II antigens of EBV-transformed B-lymphblastoid cells. I. Reduced proliferation responses of autologous and allogeneic T cells that have decreased expression of class II antigens. Hum. Immunol. 11:15.


References


