Head–head/tail–tail orientation of the pore domains of the heterodimeric ABC transporter TAP

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Head–head/tail–tail relative orientation of the pore-forming domains of the heterodimeric ABC transporter TAP

Jan C. Vos, Eric A.J. Reits, Eldine Wojcik-Jacobs and Jacques Neefjes

Background: The transporter associated with antigen processing (TAP) is a heterodimeric member of the large family of ABC transporters. The study of interactions between the subunits TAP1 and TAP2 can reveal the relative orientation of the transmembrane segments, which form a translocation pore for peptides. This is essential for understanding the architecture of TAP and other ABC transporters.

Results: The amino-terminal six transmembrane segments (TMs) of human TAP1, TAP1(1–6), and the amino-terminal five TMs of TAP2, TAP2(1–5), are thought to constitute the pore of TAP. Two new approaches are used to define dimer interactions. We show that TM6 of TAP1(1–6) is able to change topology post-translationally. This TM, along with a cytoplasmic tail, is translocated into the endoplasmic reticulum lumen, unless TAP2 is expressed. Coexpression of TM(4–5) of TAP2 stabilizes the topology of TAP1(1–6), even when the TM1 of TAP1 is subsitituted with another sequence. This suggests that the carboxy-terminal TMs of the pore-forming domains TAP1(1–6) and TAP2(1–5) interact. An alternative assay uses photobleaching in living cells using TAP1(1–6) tagged with the green fluorescent protein (GFP). Coexpression with TAP2(1–5) results in reduced movement of the heterodimer within the endoplasmic reticulum membrane, as compared with the single TAP1(1–6) molecule. In contrast, TAP2(1–4) has no effect on the mobility of TAP1(1–6)–GFP, indicating the importance of TM5 of TAP2 for dimer formation. Also, TM1 of both TAP1 and TAP2 is essential for formation of a complex with low mobility.

Conclusions: Dimerization of the pore-forming transmembrane domains of TAP1 (TM1–6) with its TAP2 counterpart (TM1–5) prevents the post-translational translocation of TM6 of TAP1 and results in a complex with reduced mobility within the endoplasmic reticulum membrane compared with the free subunit. These techniques are used to show that the pore-forming domains of TAP are aligned in a head–head/tail–tail orientation. This positions the following peptide-binding segments of the two TAP subunits to one side of the pore.

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Background

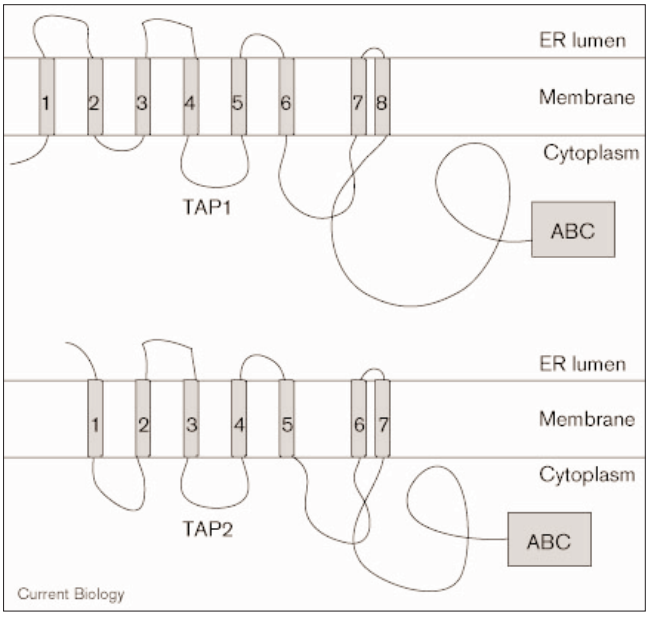
ATP-binding cassette (ABC) transporters are a large family of membrane proteins involved in the unidirectional translocation of substrates across the lipid bilayer [1]. Eukaryotic ABC transporters are characterized by two hydrophobic integral membrane domains of, usually, six transmembrane segments (TMs) and two hydrophilic nucleotide-binding domains, which provide the necessary energy for the transport process. This set of domains is either present in a single polypeptide chain or is assembled from two protein subunits. ABC transporters are involved in cystic fibrosis and also in multidrug resistance and antigen presentation. Despite a large body of genetic and biochemical data, little is known about their inter- or intramolecular interactions and the mechanism of active transport.

The transporter associated with antigen processing (TAP) is a heterodimer of two related subunits [2–5] that delivers peptides from the cytosol into the endoplasmic reticulum (ER) lumen. There, peptides associate with MHC class I molecules and are transported to the cell surface. In the absence of TAP, peptides originating in the nuclear or cytoplasmic compartments do not reach the cell surface and antigen presentation is impaired (reviewed in [6]). In the ER, TAP forms a complex with the chaperone protein tapasin and empty MHC class I molecules. Viral proteins have been identified that specifically block TAP function either by competing for peptide binding at the cytosolic side of the ER membrane or by inhibiting the transport step from the luminal side (reviewed in [7]).

We recently proposed a model for the membrane topologies of human TAP1 and TAP2 (Figure 1). We identified an unusual number of TMs, and also a cytoplasmic substrate-binding segment clearly set apart from the transmembrane pore and the nucleotide-binding domain [8]. The TAP1 subunit not only has a classical number of six TMs for what is believed to be the transmembrane pore of the transporter but, in addition, a pair of TMs that anchors the cytoplasmic peptide-binding segment to the ER membrane. In contrast, TAP2 contributes only five TMs to the putative translocation pore and has its amino terminus in the ER lumen. Like TAP1, the TAP2 peptide-binding segment has an extra pair of TMs.

We have shown by co-immunoprecipitation studies that the first six TMs of TAP1 are sufficient for dimerization with TAP2 and that further carboxy-terminal truncation abrogates stable binding to TAP2 [8]. This suggests that the complete pore-forming domain of TAP1, consisting of TM1 to TM6 (TM1–6), is necessary for a stable complex with TAP2. We present here new strategies for defining subunit interactions and especially their relative orientation. This is important for understanding the architecture of TAP and has not been defined for any of the other ABC transporters. We find that the topology of the TAP1 pore-forming domain is stabilized by TAP2 *in vivo*. In its absence, TM6 of TAP1(1–6) is translocated post-translationally into the lumen of the ER. Further dissection of the process shows that TM(4–5) of TAP2 can inhibit the conversion of TM6 of TAP1, independently of TM1 of TAP1. This observation suggests an interaction between TM6 of TAP1 and TM(4–5) of TAP2. Finally, using fusions with the green fluorescent protein (GFP) for detection we show that dimer interactions can alter the mobility of multispanning integral membrane proteins in the ER membrane in living cells. These experiments support the involvement of TM1 of both TAP1 and TAP2 in dimerization. Our data indicate that the transmembrane pore of TAP is arranged in a head–head/tail–tail orientation, positioning the peptide-binding pocket to one side of the pore.

Figure 1



Membrane topology model for the TAP1 (top) and TAP2 (bottom) subunits of human TAP. TAP1 has eight transmembrane segments (numbered TM1 to TM8), whereas TAP2 has only seven. TAP1(1–6) and TAP2(1–5) consist of the amino-terminal six and five TMs, respectively, and represent the pore-forming domains. The peptide-binding domain is located between TM6 for TAP1 (or TM5 for TAP2) and the nucleotide-binding domain (indicated as the ABC box).

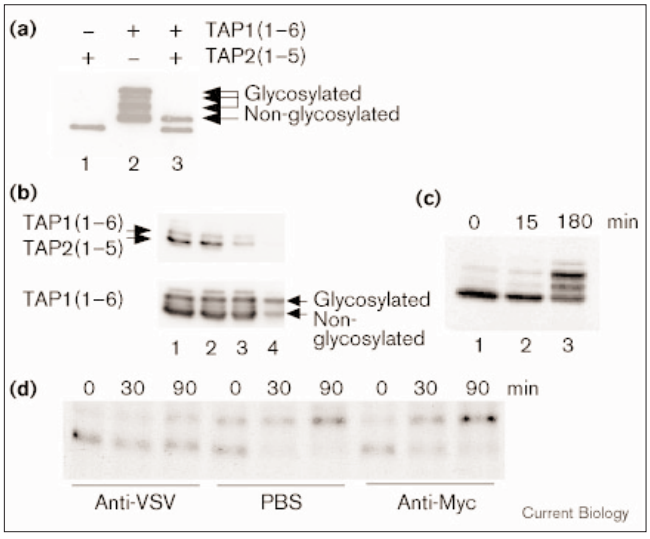
Results

Influence of TAP2 on the topology of TAP1(1–6)

Topology of the TAP subunits was studied using TAP subunit constructs truncated at the carboxy-terminal end and fused to a reporter cassette [8]. This cassette contains two potential N-linked glycosylation sites and a vesicular stomatitis virus (VSV) epitope. The glycosylation status of the chimeric protein reflects the intracellular localization of the tail, as N-glycosylation occurs exclusively in the ER lumen. The expression of TAP1(1–6) in transfected COS cells results in proteins with different glycosylation status (Figure 2a, lane 2). Although the reporter cassette contains only two N-linked glycosylation sites, we detected three bands in addition to the non-glycosylated form. This can be explained by the presence of a third glycosylation consensus sequence, which is situated at residues 227–229 immediately upstream of TM6 (residues 228–245) [2,8]. Although present in the ER lumen, this site is not glycosylated when close to a TM [9]. Therefore, the glycosylation of all three consensus sites at both sides of the TM can only be explained by a complete luminal localization of TM6. This argues against the membrane insertion of TM6 in the opposite orientation. Unexpectedly, we found that coexpression with TAP2(1–5), the counterpart of TAP1(1–6), resulted in the appearance of the non-glycosylated form of TAP1(1–6) only (Figure 2a, lane 3). This can be explained in several ways, but all indicate a direct interaction between TAP1(1–6) and TAP2(1–5). The absence of glycosylation of TAP1(1–6) in the presence of TAP2(1–5) might reflect localization of the carboxy-terminal reporter sequence in the cytoplasm, or a block of glycosylation due to steric hindrance by TAP2(1–5). Protease protection assays (Figure 2b) show equal sensitivity of TAP1(1–6) and TAP2(1–5) to trypsin digestion when both subunits are coexpressed, suggesting that their carboxyl termini are cytoplasmic (Figure 2b).

Whether glycosylation of TAP1(1–6) occurs co- or post-translationally was examined by pulse-labeling cells transfected with TAP1(1–6) for 15 minutes and chasing them for various times. SDS–PAGE of immunoprecipitated

Figure 2



Post-translational alteration of TAP1(1–6) topology. **(a)** TAP2(1–5) influences the topology of TAP1(1–6). COS cells were transfected with pTAP2(1–5) (lane 1), pTAP1(1–6) (lane 2) or a combination of both (lane 3). Extracts were prepared after 48 h and VSV-tagged TAP1 and TAP2 derivatives analyzed by 12% SDS–PAGE. Proteins were transferred to nitrocellulose and probed with anti-VSV antibodies. Enhanced chemoluminescence (ECL) reactions were visualized by autoradiography. The positions of the glycosylated and non-glycosylated forms of TAP1(1–6) are indicated. **(b)** Protease protection assay. COS cells were transfected with pTAP1(1–6) and pTAP2(1–5) (top panel) or with pTAP1(1–6) alone (bottom panel). Microsomes were prepared after 48 h and treated without trypsin (lanes 1) or with 0.5 μg/ml (lanes 2), 1.5 μg/ml (lanes 3) or 5 μg/ml (lanes 4) trypsin for 10 min at 20°C in PBS. The luminal ER protein disulfide isomerase is not affected by trypsin under these conditions (data not shown). Reaction products were analyzed as in (a). The positions of TAP1(1–6) and TAP2(1–5) are indicated by arrows (top panel), as well as the main glycosylated and non-glycosylated TAP1(1–6) polypeptides (bottom panel). **(c)** Post-translational glycosylation of TAP1(1–6). COS cells were transfected with pTAP(1–6), pulse-labeled and chased for the times indicated. Proteins were precipitated with anti-VSV antibodies, separated by 12% SDS–PAGE and visualized by autoradiography. **(d)** *In vitro* post-translational translocation of TM6 of pTAP1(1–6) is inhibited by specific antibodies against the carboxyl terminus. TAP1(1–6) was translated in a reticulocyte lysate in the presence of microsomes for 15 min. Isolated microsomes were chased in PBS at 30°C for the times indicated in the absence (PBS) or presence of specific (anti-VSV) or control (anti-Myc) antibodies. Proteins were separated by 12% SDS–PAGE and visualized by autoradiography.

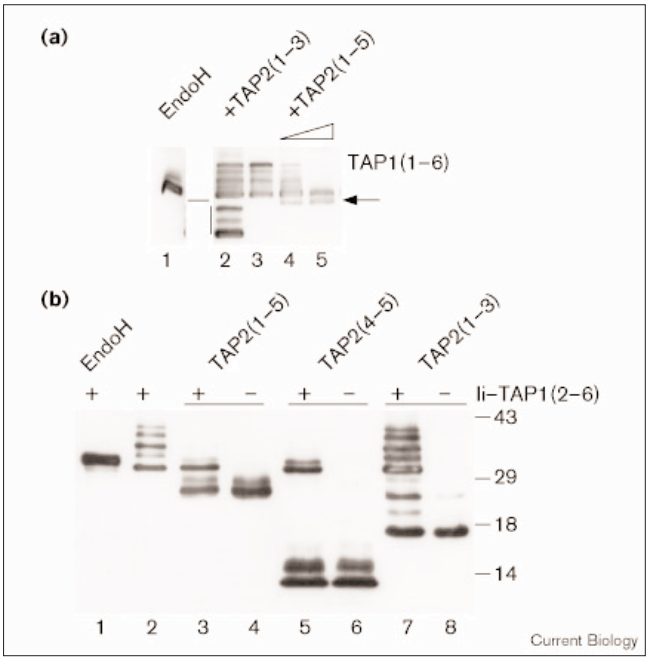
TAP1 species showed that the non-glycosylated forms were converted with time into the glycosylated forms (Figure 2c). Whereas glycosylation is considered to be a co-translational event, this suggests post-translational translocation of TM6 followed by glycosylation. Delayed glycosylation could, however, be an alternative explanation.

In vitro translation was used to investigate this further. TAP1(1–6) was synthesized in a reticulocyte lysate containing canine microsomes, followed by a chase period in phosphate-buffered saline (PBS). As in the *in vivo* situation, the initial membrane insertion of TAP1(1–6) results in a mainly non-glycosylated form, which converts into a glycosylated form during the chase period following *in vitro* translation (Figure 2d, PBS), consistent with a post-translational event. Washing the membranes before the chase period with 10 mM EDTA in PBS to eliminate translation-arrested ribosomes does not alter the outcome (data not shown). To test whether the non-glycosylated initial product of TAP1(1–6) has its carboxyl-terminus in the cytosol, an antibody against the VSV epitope at the extreme carboxyl terminus of the protein was added during the chase period. The anti-VSV antibody interferes with the appearance in time of the glycosylated form, whereas a control antibody (anti-Myc) has no effect. Thus, binding of the antibody at the carboxyl terminus of a fully synthesized TAP1(1–6) molecule at the cytosolic face of the membrane prevents its post-translational translocation across the ER membrane. We conclude that TAP1(1–6) is *de novo* inserted into the membrane with its carboxyl terminus in the cytoplasm, as indicated in Figure 1. In the absence of TAP2, TM6 is post-translationally translocated into the ER lumen. Whether the translocon is involved, as has been shown for retrograde transport from the ER [10–12], remains to be determined.

Evidence for a tail–tail configuration of the TAP pore

The TMs of multi-spanning integral membrane molecules are inserted into the membrane sequentially (reviewed in [13]). Therefore, a plausible orientation of the two similar halves of ABC transporters would be that the sixth TM of the first half would line up with the first TM of the second half (tail–head). Alternatively, the TMs of each subunit could interact in a head–head/tail–tail configuration. We used the post-translational conversion of the membrane topology of TM6 of TAP1(1–6) as readout for association with the pore-forming domain of TAP2. COS cells transfected with TAP1(1–6) as well as similarly VSV-tagged TAP2 constructs were analyzed by western blotting. As shown above, TAP2(1–5) maintains TAP1(1–6) in the non-glycosylated form, and this occurs in a dose-dependent manner (Figure 3a). In contrast to TAP2(1–5), TAP2(1–3) is not able to stabilize TAP1(1–6) in its original topology. This suggests that TM4 and/or TM5 of TAP2 interact with TM6 of TAP1. To confirm these observations, other combinations were tested (Figure 3b). We substituted TM1 of TAP1 by an invariant chain (Ii) TM to investigate the role of TM1 in the stabilization of TM6 by TAP2(1–5) (Figure 3b, compare lanes 2 and 3). A similar result was obtained as with TAP1(1–6), showing that TM1 of TAP1 is not essential for stabilization. Note that the Ii–TAP1(2–6) construct has an additional glycosylation site at residue 250 [2] compared with TAP1(1–6), because the carboxy-terminal TAP1 amino acid in Ii–TAP1(2–6) is His257 instead of Gly247 in TAP1(1–6). Again, coexpression of TAP2(4–5) is sufficient to prevent glycosylation (Figure 3b, lane 5). This indicates a direct interaction

Figure 3



TM4 and TM5 of TAP2 are able to stabilize TM6 of TAP1(1–6), independent of TM1 of TAP1. **(a)** COS cells were transfected with pTAP1(1–6) either alone (lane 3), in combination with pTAP12(1–3) (lane 2) or with increasing amounts of pTAP2(1–5) (lanes 4,5). Extracts were prepared after 48 h and VSV-tagged TAP1 and TAP2 derivatives were analyzed by 12% SDS–PAGE. Proteins were transferred to nitrocellulose and probed with anti-VSV antibodies. ECL reactions were visualized by autoradiography. Lane 1 is the result of endoglycosidase H (EndoH) digestion of the extract shown in lane 3. The horizontal bar between lanes 1 and 2 indicates the position of the 29 kDa molecular-weight marker. TAP2(1–5) is marked with an arrow, while TAP2(1–3) products run at the positions indicated with the vertical bar. **(b)** COS cells were transfected with pIi–TAP1(2–6) (lanes 1–3,5,7) and/or TAP2 derivatives as indicated at the top of the figure and proteins were analyzed as in (a). The positions of molecular-weight markers are indicated on the right.

between TM6 of TAP1 and TM4–5 of TAP2, supporting a tail–tail orientation for the pore of TAP. The alternative head–tail model is incompatible with these data.

Endoplasmic reticulum mobility studies indicate head–head interactions

The biochemical data obtained above suggest that the TAP subunits can diffuse freely within the ER membrane to form a complex. To investigate the mobility of a multi-spanning integral membrane molecule like TAP1(1–6), either alone or in complex with TAP2, we made use of GFP, a fluorescent marker protein that can be visualized in living cells. ER mobility of TAP–GFP fusions was compared with that of free GFP and of GFP coupled to a single TM, the Type 1 signal and anchor of TAP2 [8].

Mobility was determined by photobleaching a small region of the cell, which results in the irreversible inactivation of GFP [14,15]. The recovery of fluorescence is dependent on the movement of proteins within the plane of the membrane from the area surrounding the bleached box and can be quantitated. Figure 4a shows this for the various constructs. Free GFP is located within the cytoplasm and nucleus and diffuses rapidly. GFP fused to either one or six TMs, and thus inserted in the ER membrane, diffuses more slowly. The mobility of GFP linked to either one (TAP2(1)–GFP) or six TMs (TAP1(1–6)–GFP) is comparable, suggesting that the molecular weight or the number of TMs is not a major determinant of mobility. This is consistent with theoretical models for protein diffusion in membranes [16] as well as experimental data [17,18].

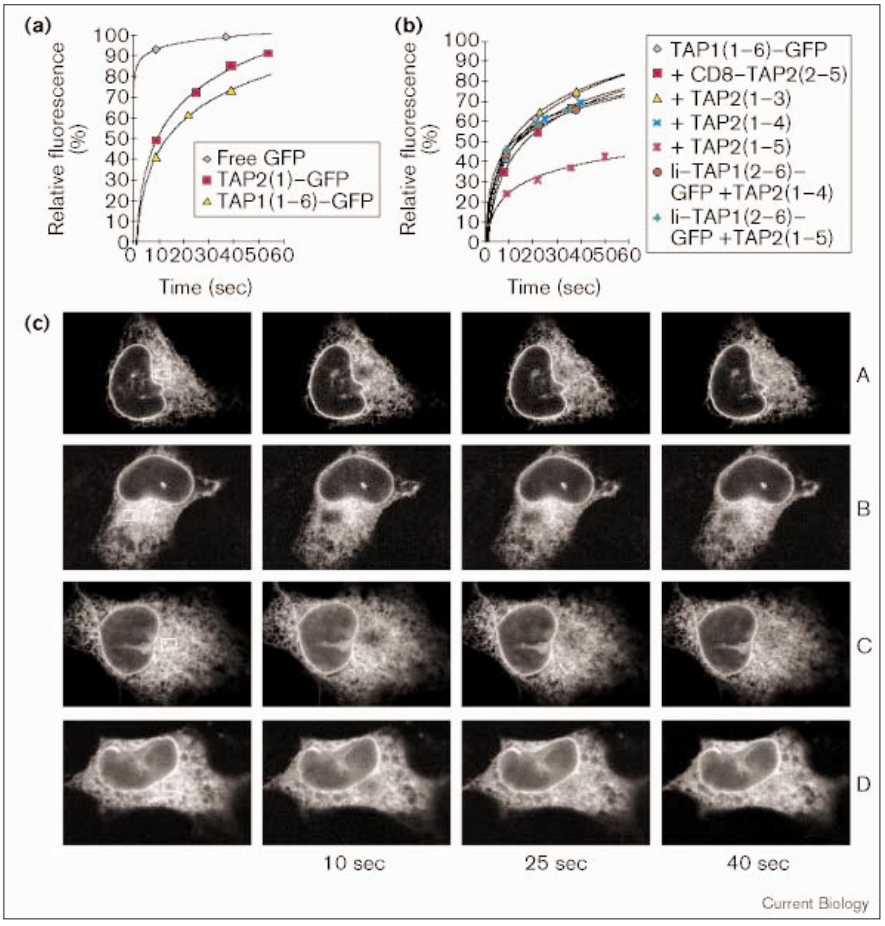
Given that the radius of the transmembrane portion of a protein is a major determinant of its mobility in the membrane [16], we tested the effect of complex formation with excess TAP2(1–5) on the mobility of TAP1(1–6)–GFP. Photobleaching experiments show a reduced mobility for the complex of TAP1(1–6)–GFP and TAP2(1–5) (Figure 4b,c, compare A and B), which is probably the result of pore formation. To analyze which TMs of TAP2 are required for this reduction in mobility, several TAP2 derivatives were coexpressed with TAP1(1–6)–GFP. Comparable expression levels of TAP2 derivatives were confirmed by western blot (data not shown). The inability of TAP2(1–4) to reduce the mobility of TAP1(1–6)–GFP indicates that TM5 of TAP2 is involved in dimerization. Furthermore, construct CD8–TAP2(2–5), in which TM1 is substituted with the transmembrane and extracellular segment of CD8, is also unable to reduce the mobility of TAP1(1–6)–GFP. These findings suggest that both the amino- and carboxyl terminal TMs of the pore-forming domain of TAP2 are required to change the ER mobility of the TAP1 subunit. To test whether TM1 of TAP1 is also involved in subunit interactions, a TAP1 derivative with an altered TM1, Ii–TAP1(2–6), was tagged with GFP. Addition of TAP2(1–5) had no effect on the mobility of this TAP1 construct, demonstrating that TM1 of TAP1 is involved in the formation of a pore with reduced mobility (Figure 4b).

Together with the results of the post-translational translocation assays, these experiments suggest a head–head/tail–tail orientation of the pore with direct involvement of TM1 and TM6 of TAP1 and TM1 and TM5 of TAP2.

Discussion

Two new approaches to studying the subunit interactions between TAP1 and TAP2 have resulted in a model for the ABC transporter showing a head–head/tail–tail orientation (Figure 5). Very little is known about the relative orientation of the two halves of other ABC transporters. One study, using oxidative cross-linking between engineered cysteine residues, supports the proximity of TM6 and 12 in the ABC family member P-glycoprotein [19], giving a

Figure 4



Fluorescence recovery. **(a)** Fluorescence recovery of free GFP, TAP2(1)–GFP and TAP1(1–6)–GFP. The lines show recovery of fluorescence in photobleached regions in the ER of COS cells transfected with constructs as indicated. **(b)** Fluorescence recovery of TAP1(1–6)–GFP or Ii–TAP1(2–6)–GFP with or without different TAP2 constructs. **(c)** Fluorescence photobleaching recovery of TAP1(1–6)–GFP with and without TAP2 constructs. Living COS transfectants were analyzed by confocal microscopy at 37°C. TAP1(1–6)–GFP shows a characteristic ER localization: fluorescence in the nuclear envelope and a fine reticular network. A small region of the ER (indicated by the box) was photobleached immediately after the first image by scanning the region for 10 sec. Recovery of fluorescence in the photobleached region is shown by the images collected after photobleaching at the times indicated. In (A) the photobleached region of a cell transfected with TAP1(1–6)–GFP rapidly recovered fluorescence, whereas in (B) the region of a cell transfected with TAP1(1–6)–GFP and TAP2(1–5) recovered much more slowly. Recovery in cells transfected with TAP1(1–6)–GFP together with TAP2(1–4) (C) or CD8–TAP2(2–5) (D) is comparable to TAP1(1–6)–GFP only.

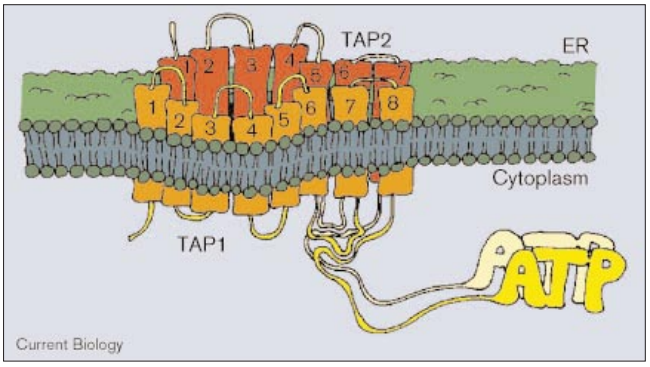
mirror-symmetry model for the two related halves of P-glycoprotein analogous to the TAP model presented here.

Initially, TAP1(1–6) is inserted into the ER membrane according to the model presented in Figure 1. In the absence of TAP2, however, TM6 of TAP1(1–6) is unstably embedded in the membrane. This is a unique and peculiar feature of this construct and there is no indication that TM6 is unstable in the full-length molecule, because consensus N-linked glycosylation sites close to TM6 and one in the loop downstream of TM6 are not used. The complete translocation of TM6 of TAP1 occurred both *in vivo* and *in vitro* and does not require exogenous ATP (Figure 2c). The binding of an antibody to the cytoplasmic tail of the molecule prevents translocation. This implies that the free carboxy-terminal glycosylation tag is transported across the membrane post-translationally. Several proteins have been reported to have multiple topological orientations (reviewed in [20]), but none of these examples is the result of a post-translational alteration. Rather, the topology is determined by *cis*- and *trans*-acting factors during co-translational translocation, as shown recently for the prion protein [21]. Another study reports on retrograde movement through the translocon during the process of translation [22]. Here, glycosylation or protein folding of the translocated chain could achieve unidirectionality. This might explain the accumulation of the glycosylated form of TAP1(1–6).

The post-translational translocation has been used to determine subunit interactions between TAP1 and TAP2. Translocation of TM6 of TAP1(1–6) is prevented by the expression of TM(4–5) of TAP2. Because TM1 of either TAP1 and TAP2 is not required for this interaction to occur, this indicates that the pore-forming segments of TAP1 (TM1–6) and of TAP2 (TM1–5) are connected in a tail-tail orientation. An indirect interaction between TAP1 and TAP2 is unlikely, because on purification from insect cells, TAP behaved as a heterodimer when analyzed by size-exclusion chromatography [23].

The study of the ER mobility of TAP1(1-6)–GFP fusions has revealed further interactions between the two TAP subunits. The crucial observation was that the association of TAP1(1–6) and TAP2(1–5) resulted in a complex of

Figure 5



Model of the TAP heterodimeric complex. The TAP complex consists of three domains: a transmembrane pore in a head–head/tail–tail orientation, a peptide-binding domain and the nucleotide-binding domain (shown here as ATP). The peptide-binding segment is made up of interacting segments from the two subunits, is anchored in the ER membrane by two TM doublets and is located between the pore region and the two nucleotide-binding domains (see also [8]). The tail–tail orientation positions these segments to one side of the pore.

lower mobility compared with the free subunit. This could be the result of the assembly of a complex containing additional proteins, such as chaperones. It seems unlikely that formation of a complete pore is accompanied by the association with molecules involved in MHC class I presentation, because we (over-)express the molecules in COS cells and furthermore, proteins like tapasin and MHC class I can already bind to the single TAP subunits [24]. A more likely explanation would be that the assembly of a circular pore reduces the translational mobility of the complex because of an increased radius. Notably, the diffusion coefficient of a lipid embedded protein complex has been shown to be related to the radius and not to the mass [16]. Disruption of either head–head or tail–tail interactions does not abolish remaining dimer interactions, but results in a complex with a mobility comparable to the free TAP1(1–6)–GFP subunit, because the pore is not maintained and the radius of the transmembrane domain is reduced.

In conclusion, the picture that has emerged for TAP is summarized in Figure 5. The pore is composed of six TMs of TAP1 and five TMs of TAP2 with a head–head and tail–tail-orientation. The segments downstream of the pore contain an additional pair of TMs that are part of the peptide-binding domain that heterodimerizes [8] and are located on the same side of the pore. It is possible that ATP hydrolysis induces conformational changes that introduce these TM doublets into the 'closed' pore. This transition would then automatically open the pore and, at the same time, position the cytoplasmic peptide-binding domain under the opened pore. This model couples peptide translocation to pore opening. Whereas our model does not involve additional TM interactions in the pore-forming domains, we can not formally exclude them. However, the structure of P-glycoprotein at 2.5 nm resolution shows a central pore consistent with a simple head–head/tail–tail orientation [25].

Conclusions

The integral membrane pore-forming domains of the two TAP subunits are arranged in a head–head/tail–tail orientation; both the amino- and carboxy-terminal TMs are juxtaposed in the heterodimer complex. This has important implications for the positioning of the substrate-binding domain that now locates to one side of the pore. Dimer formation prevented the post-translational alteration of the topology of the pore-forming domain of TAP1. ER mobility studies can be used to study conformational changes within integral membrane proteins.

Materials and methods

Transfections and extract preparation

COS-7 cells were maintained in DMEM supplemented with 7.5% fetal calf serum. Transfections were performed using the DEAE–dextran method. Cells were harvested in PBS, cell pellets lysed in 50 mM Tris pH 7.5, 1 mM EDTA, 150 mM NaCl, 1% NP-40 (TEN/NP40) on ice for 30 min. Digestion with endoglycosidase H (Boehringer, Mannheim) was done at 50 μU/μl for 3 h at 37°C in lysis buffer supplemented with 0.2% SDS and 1 mM phenylmethansulfonyl fluoride (PMSF). Membranes were prepared as described [8].

DNA constructs

Truncations of TAP1 and TAP2 have been generated in which the carboxyl terminal amino acid is used to name the construct [8]. Plasmid B1.G247 was obtained by cloning the *Xba*I fragment of pTAP1–G247 into pBluescript. pTAP(1–6) is equivalent to pTAP1–G247. Similarly, pTAP2(1–5) is identical to pTAP2–R210, pTAP2(1–4) to pTAP2–R175 and pTAP2(1–3) to pTAP2–E128. pTAP2(4–5) was obtained by standard PCR technology using a 5′ primer to start at amino acid S120 in combination with 3′ primer R210 and subsequent cloning into the *Sal*I-site of pMT2IiVSV [8].

pTAP1(1–6)–GFP was obtained by cloning the *Sal*I fragment of pTAP1–H257 into pEGFP–N3 (Clontech). pTAP2(1) was obtained by cloning the *Sal*I–*Not*I fragment of pTAP2–R45 into pEGFP–N3. pIi-TAP1(2–6)–GFP was obtained by cloning the *Pst*I–*Sal*I fragment obtained after a partial *Pst*I-digestion of pIi-TAP1(2–6) into pEGFP–N3.

In vitro *transcription and translation*

RNA was prepared from pB1.G247 linearized with *Sac*I according to the protocol provided by Promega. *In vitro* translations were performed as suggested by the manufacturer in rabbit reticulocyte lysates supplemented with microsomes prepared from dog pancreas (Promega) and [^{35}S]methionine/cysteine (Amersham, 10 mCi/ml). After a 15 min pulse at 30°C, lysates were cooled to 4°C and membranes were pelleted for 20 min at 14,000 rpm, washed with ice-cold PBS, and chased in PBS at 30°C for the times indicated. Antibodies (1 μl; anti-VSV-G–peroxidase or anti-Myc–peroxidase, Boehringer) was added per 0.5 μl microsomes during the chase.

Pulse–chase experiments

COS-7 cells were starved for 30 min, 48 h after transfection with pTAP1–G247, in Met/Cys-free RPMI medium including 10% FCS followed by labeling the cells with 80 μCi [^{35}S]Met/Cys for 15 min. The pulse was stopped by addition of non-radioactive Met/Cys to a final concentration of 1 mM. After culturing for various times, cells were lysed in lysis mixture and the TAP species were immunoprecipitated with the anti-VSV antibody P5D4 prior to analysis by 12% SDS–PAGE.

Confocal analysis and photobleaching

Living cells were analyzed in a tissue-culture device at 37°C using a 600MRC confocal microscope equipped with an argon/krypton laser (BioRad Labs, Hercules, CA). Green fluorescence was detected at $\lambda > 515$ nm after excitation at $\lambda = 488$ nm. To bleach GFP-tagged TAP1(1–6) in living cells, a small region of the ER was scanned for 10 sec.

Fluorescence recovery after photobleaching

To investigate mobility of GFP-tagged TAP1(1–6), fluorescence recovery after photobleaching (FRAP) experiments were performed [14,15]. A box in the ER was photobleached and the effect on the fluorescence in this box versus a box in the ER outside the bleached region was quantitated. From these data, recovery of fluorescence by diffusion of unbleached GFP-tagged TAP1(1–6) after bleaching can be determined using the formula % recovery = (FA after + FB before – FB after)/(FA before). FA is the fluorescence in the bleach box and FB the fluorescence in the box outside this region. The fluorescence is determined before and after photobleaching, and both FA and FB will decrease slightly due to equal photobleaching of the complete cell during imaging.

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