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Research Article

Identification of domain boundaries within the N-termini of TAP1 and TAP2 and their importance in tapasin binding and tapasin-mediated increase in peptide loading of MHC class I

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Summary Before exit from the endoplasmic reticulum (ER), MHC class I molecules transiently associate with the transporter associated with antigen processing (TAP1/TAP2) in an interaction that is bridged by tapasin. TAP1 and TAP2 belong to the ATP-binding cassette (ABC) transporter family, and are necessary and sufficient for peptide translocation across the ER membrane during loading of MHC class I molecules. Most ABC transporters comprise a transmembrane region with six membrane-spanning helices. TAP1 and TAP2, however, contain additional N-terminal sequences whose functions may be linked to interactions with tapasin and MHC class I molecules. Upon expression and purification of human TAP1/TAP2 complexes from insect cells, proteolytic fragments were identified that result from cleavage at residues 131 and 88 of TAP1 and TAP2, respectively. N-Terminally truncated TAP variants lacking these segments retained the ability to bind peptide and nucleotide substrates at a level comparable to that of wild-type TAP. The truncated constructs were also capable of peptide translocation *in vitro*, although with reduced efficiency. In an insect cell-based assay that reconstituted the class I loading pathway, the truncated TAP variants promoted HLA-B*2705 processing to similar levels as wild-type TAP. However, correlating with the observed reduction in tapasin binding, the tapasin-mediated increase in processing of HLA-B*2705 and HLA-B*4402 was lower for the truncated TAP constructs relative to the wild type. Together, these studies indicate that N-terminal domains of TAP1 and TAP2 are important for tapasin binding and for optimal peptide loading onto MHC class I molecules.

Key words: ATP-binding cassette (ABC) transporter, antigen presentation, tapasin, transporter associated with antigen processing (TAP).

Introduction

ATP-binding cassette (ABC) transporters translocate a variety of substrates, ranging from small molecules to whole proteins, across membranes.[1] ABC transporters share a common domain organization, with two transmembrane domains (TMD) and two cytoplasmic ABC-type nucleotide binding domains (NBD), and they use the energy of ATP binding and hydrolysis to select and transport the substrates. Well studied ABC transporters include the bacterial histidine transporter ($HisJQMP_2$) and maltose transporter ($MalEFGK_2$), which use a periplasmic substrate-binding protein (HisJ or MalE) to import their substrate,[2] and mammalian ABC transporters (essentially all substrate exporters), including the multidrug resistance protein P-glycoprotein, the cystic fibrosis transmembrane conductance regulator (CFTR), and the transporter associated with antigen processing (TAP).

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The ABC transporter associated with antigen processing, TAP, transports cytosolic peptides generated by the proteasome into the endoplasmic reticulum (ER) for loading onto MHC class I molecules (reviewed in van Endert *et al*. 2002[3]). Once loaded, class I molecules travel to the cell surface and present the peptides to T cells. This enables the immune system to recognize and eliminate deregulated or cancerous cells, virally infected cells and foreign cells (e.g. graft rejection). The active TAP transporter is composed of two homologous subunits: TAP1 and TAP2.

A common set of four domains is shared by ABC transporters: two homologous TMD and two homologous ATPase or NBD. These four domains can assemble from different gene combinations ranging from four different polypeptides to a single polypeptide.[1] A number of ABC transporters have additional domains. For example, most bacterial ABC transporter import systems have accessory periplasmic proteins (e.g. MalE or maltose-binding protein [MBP] for maltose transport). Another example is the CFTR regulatory domain inserted between the first NBD and the second membrane-spanning domain. A third example is the multidrug resistance protein MRP1, which has an additional N-terminal domain

consisting of five putative transmembrane (TM) helices. TAP also contains an N-terminal extension in both TAP1 and TAP2, which is predicted to contain three or four membrane-spanning helices by hydrophobicity analyses. Although poorly conserved in sequence, our analyses indicate this extension is similar in length and hydrophobic character to a few other ABC transporters, all of which are involved in peptide or polypeptide export. Over the years there have been conflicting reports on the functional breakdown of the TAP protein's membrane-spanning helices.[4–6]

In addition to TAP, MHC class I loading also requires ER-resident chaperones, including the class I-specific chaperone tapasin. Tapasin comprises an N-terminal ER lumenal domain that associates with MHC molecules, followed by a C-terminal TM and cytosolic stalk that bind TAP.[7] Currently, it is thought that tapasin retains MHC class I in the ER until bound to a high affinity peptide. Loss of tapasin reduces surface class I expression or leads to suboptimal class I-peptide complexes, depending on the experimental system.[8] Tapasin has also been shown to increase TAP stability, and by linking TAP to MHC class I it may act to increase the local peptide concentration for efficient loading.[9–11]

Materials and methods

Cell culture and infection

Sf21 insect cells (*Spodoptera frugiperda*) were cultured in Hink's TNM-FH media supplemented with 10% foetal bovine serum (FBS) and 0.1% pluronic F-68 (Mediatech, Herndon, VA, USA). Baculovirus stocks were prepared according to the Bac-to-Bac Baculovirus Expression System (Invitrogen, Carlsbad, CA, USA). Expression of protein was determined by SDS–PAGE and immunoblotting with anti-His (C-term)/AP (Invitrogen), anti-TAP1 (monoclonal 1.28[12]), anti-TAP2 (monoclonal 2.17[12] or polyclonal R.RING11C[13]), anti-tapasin (rabbit polyclonal R.gp48N[14]) and anti-H chain (monoclonal HC10[15]). Where necessary, AP-rec-protein G (Zymed, San Francisco, CA, USA) was used as a secondary antibody.

Cloning of constructs

For wild-type TAP (WT-TAP) expression in insect cells, human TAP1 (with a C-terminal 6xHis-tag introduced by PCR amplification [5′ primer CGCGGCAGCCATATGGCTAGCTCTAGGTGTCC; 3′ primer AGCCGGATCCTTAATGATGATGATGATGATGTTCTGGAGCATCTGCAGGAG] followed by *Nde*I/*Bam*HI digestion and Klenow fill-in) was cloned into the *Sma*I site and human TAP2 (5′ primer AGCCAGATCTATGCGGCTCCCTGACCTG; 3′ primer AGCCAGATCTTTAGAGCTGGGCAAGCTTCTGC; digested with *Bgl*II) was cloned into the *Bam*HI site of pFastBac Dual (Invitrogen). The *Bam*HI site within the TAP2 cDNA was then removed through a silent mutation using QuickChange (Stratagene, La Jolla, CA, USA). To generate 7TM-TAP, human TAP1 (125–746; C-terminal 6xHis-tag; 5′ primer AGCCGGATCCATGGCGGATAGCACCAGGCTAC; 3′ primer AGCCCTCGAGTTAATGATGATGATGATGATGTTCTGGAGCATCTGCAGGAG) was introduced into the *Bbs*I and *Xho*I sites of pFastBac Dual and human TAP2 (87–686; 5′ primer AGCCGGATCCATGTCACGTGCTCCCCCAGCC; 3′ primer AGCCTCTAGATTAGAGCTGGGCAAGCTTCTGC) was cloned into the *Bam*HI and *Xba*I sites. An analogous strategy was used for 6TM-TAP (TAP1 174–746 [5′ primer AGCCGGATCCATGCGTCGGCTTCTAGGCTG]; TAP2 138–686 [5′ primer AGCCGGATCCATGTGGAGGCTGCTGAAGC]). For HLA-B*2705 expression, the heavy chain open reading frame (5′ primer AGCCGGATCCATGCGGGTCACGGAGCC; 3′ primer CAGCCCTCGAGTCAAGCTGTGAGAGACACAT) was cloned into the *Bbs*I and *Xho*I sites of pFastBac Dual while the β_2-microglobulin open reading frame was cloned into the *Bam*HI and *Xba*I sites. For HLA-B*4402 expression, PCR was used to engineer *Bam*HI sites at the 5′ and 3′ ends of the sequence (5′ primer TTGGATCCCCACCATGCGGGTCACGGC; 3′ primer ATGGATCCTCAAGCTGTGAGAGA). The PCR product was ligated into pCRscript (Stratagene), sequenced, and then excised and ligated into the *Bam*HI site of pAcUW31 baculovirus transfer vector that had a DNA sequence encoding chimp β_2-microglobulin ligated into the *Bgl*II site to generate pAcUW31-B*4402. The full-length tapasin open reading frame[16] was digested from pCR2.1 with *Kpn*I and *Cla*I; the resulting fragment was treated with T4 DNA polymerase to fill in the restriction overhangs and cloned into the *Stu*I site of pFastBac1. All pFastBac clones were confirmed by sequencing then transformed into DH10Bac *Escherichia coli* cells (Invitrogen) to produce Bacmid viral DNA, and transfected into Sf9 or Sf21 cells to generate baculoviruses, following the Bac-to-Bac system instructions (Invitrogen). Virus stocks were amplified and titred by plaque assays. pAcUW31-B*4402 was co-transfected with BaculoGold DNA (BD Biosciences, San Jose, CA, USA) into insect cells, according to the manufacturer's instructions. Pure virus was isolated using plaque assays and further amplified by re-infection.

Purification of the TAP N-terminal proteolysis products

Wild-type TAP-infected Sf21 cells were harvested 96 h post-infection and lysed in 20 mmol/L Tris-HCl (pH 8.0), 100 mmol/L NaCl, 20% glycerol, 5 mmol/L $MgCl_2$, 2 mmol/L ATP, 2.5 mmol/L benzamidine, 1 mmol/L PMSF and 1% dodecyl-β-D-maltopyranoside (Anatrace, Maumee, OH, USA). After centrifugation at 100 000 *g*, the supernatant was incubated with Ni-NTA agarose (Qiagen, Valencia, CA, USA). The resin was washed with 20 mmol/L Tris-HCl (pH 8.0), 20 mmol/L imidazole, 100 mmol/L NaCl, 20% glycerol, 5 mmol/L $MgCl_2$, 2.5 mmol/L benzamidine, 1 mmol/L PMSF and 0.1% dodecyl-β-D-maltopyranoside, and eluted with 20 mmol/L Tris-HCl (pH 8.0), 300 mmol/L imidazole, 100 mmol/L NaCl, 20% glycerol, 5 mmol/L $MgCl_2$, 2.5 mmol/L benzamidine, 1 mmol/L PMSF and 0.1% dodecyl-β-D-maltopyranoside. The eluted sample was separated on a 10% low-bis SDS–polyacrylamide gel (1:200 bis-acrylamide to acrylamide ratio), transferred onto a polyvinylidene fluoride (PVDF) membrane and the bands of interest were submitted for N-terminal sequencing at the MIT-HHMI Biopolymers Laboratory (Cambridge, MA, USA) to identify their N-terminal proteolysis sites.

In vitro *peptide binding and transport assays*

Peptide binding was quantified using fluorescence quenching assays with a fluorescein-conjugated RRYQKCTEL peptide, as previously described.[17,18] The transport assay measured translocation of ^{125}I-labelled RRYNASTEL peptide into microsomes and is also described elsewhere.[17]

Nucleotide binding assays

Infected Sf21 cells (1×10^7) were lysed at 4°C in 1 mL solubilization buffer (10 mmol/L Tris [pH 7.5], 10 mmol/L sodium phosphate, 130 mmol/L NaCl, 5 mmol/L $MgCl_2$, 2.5 mmol/L benzamidine, 1 mmol/L PMSF and 1% dodecyl-β-D-maltopyranoside) for 30 min, after which insoluble particles were removed by centrifugation (18 000 *g*, 20 min). Nucleotide-agarose beads (75 μL; 9-atom linker to C-8; Sigma, St Louis, MO, USA) resuspended in equilibration

buffer (100 mmol/L Tris [pH 7.5], 500 mmol/L NaCl) were added to the soluble lysate. Binding proceeded at 4°C for 1 h. The beads were washed three times with 1 mL solubilization buffer (0.33% dodecyl-β-D-maltopyranoside) and resuspended in SDS–PAGE sample buffer.

Insect cell-based MHC class I loading assay

This assay was adapted from Lauvau *et al*. 1999.[19] Briefly, 5×10^5 Sf21 cells were harvested 48 h post-infection, washed with PBS/1% FBS and stained for 30 min with a 1:10 dilution of W6/32-FITC conjugate (Sigma) in 50 μL cold PBS/1% FBS. Stained cells were washed with PBS/1% FBS before data collection on a FACSCalibur flow cytometer (BD Biosciences). Data was analysed using CellQuest Pro (BD Biosciences).

Tapasin binding assay

TAP was precipitated with ADP-agarose as described above, except that dodecyl-β-D-maltopyranoside was replaced with 1% digitonin during lysis and 0.3% digitonin during washes. Co-precipitation of tapasin and MHC was determined by western blot analysis.

Results

TAP1 and TAP2 were proteolysed in insect cells

Wild-type human TAP (WT-TAP) was expressed in insect cells using a dual expression baculovirus containing full-length TAP1 with a C-terminal 6-His tag and full-length untagged TAP2. Expression was monitored over the course of 96 h by western blot analysis (Fig. 1A). Both proteins were expressed by 48 h post-infection. Weaker bands of lower molecular weight appeared from 72 h and increased in intensity over the time course. These low molecular weight products were detected with antibodies targeting the C-termini of TAP1 (anti-His) and TAP2 (R.RING11C polyclonal antibody directed against the C-terminal residues of TAP2A[13]), indicating protease-sensitive sites near the N-termini. After purification of the dodecylmaltoside-solubilized TAP heterodimer by Ni-NTA chromatography, the proteolysis products were separated by low-bis SDS–PAGE and identified by N-terminal sequencing. The fragments resulted from cleavage at residues 131 and 88 of TAP1 and TAP2, respectively. TAP2 was also proteolysed to a lesser degree at a second internal site that was not identified. The proteolytic cleavages presumably arise from exposure of loops separating TM segments that were previously protected from proteases through cellular compartments, which break down during baculovirus-infected insect cell death and release proteases. Based on previous membrane topology studies and prediction algorithms, these protease sensitive sites are expected to lie in ER lumenal loops of their respective proteins (Fig. 1B).

Protease-sensitive sites often reside in the exposed linkers that separate structural modules or domains of proteins. We therefore decided to investigate the biochemical properties of a TAP heterodimer missing residues N-terminal of the proteolysis sites. Each subunit of this truncated TAP dimer is predicted to have seven TM helices, hence this construct is referred to as 7TM-TAP (Fig. 1B). In addition, homology to other ABC transporters suggested a core conserved TM domain of only the last six membrane-spanning helices. We therefore generated a second construct beginning at residue

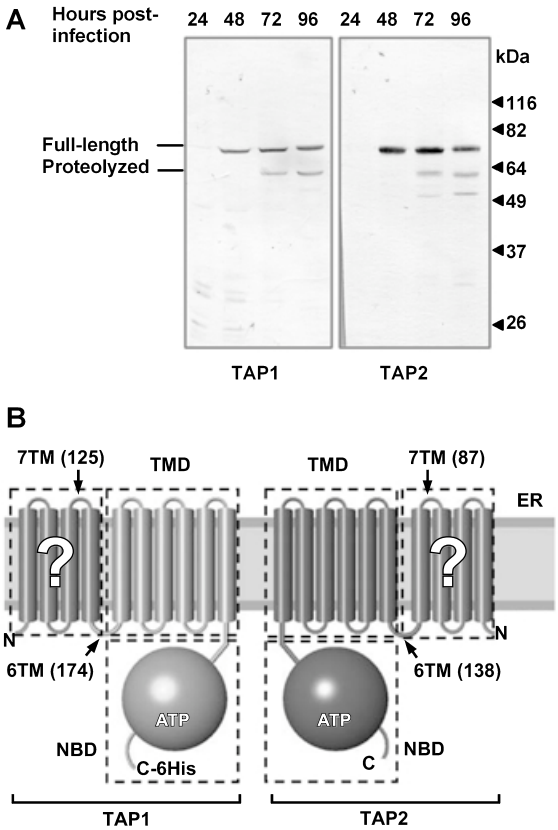

Figure 1 (A) TAP1 and TAP2 proteolysis in baculovirus-infected insect cells. Full-length TAP1 and TAP2 were expressed in Sf21 cells using baculovirus infection. Expression was detected by SDS–PAGE and western blotting, with equal cell numbers loaded in each lane. Proteolysis of both TAP1 and TAP2 was observed. (B) Proposed topology diagram of TAP1 and TAP2. Using N-terminal sequencing, the approximately 60 kDa proteolytic products were found to start at residue 131 of TAP1 and 88 of TAP2. Based on these proteolysis sites and sequence identity to other ABC transporters, two deletion constructs were designed as shown: 7TM-TAP (TAP1 [125–746]/TAP2 [87–686]) and 6TM-TAP (TAP1 [174–746]/TAP2 [138–686]). ER, endoplasmic reticulum; NBD, nucleotide binding domain; TM, transmembrane; TMD, transmembrane domain.

174 of TAP1 and 138 of TAP2, which is referred to as 6TM-TAP (Fig. 1B). All constructs include a C-terminal 6xHis-tag on TAP1.

7TM- and 6TM-TAP bind nucleotides and peptide

The N-terminal deletion constructs were first analysed for their ability to bind substrate. Using agarose beads conjugated to specific adenosine nucleotides, it was shown that solubilized WT-, 7TM- and 6TM-TAP could be precipitated by ADP- and ATP-agarose, but not by AMP-agarose (Fig. 2A). In each case, the binding to ATP-agarose could be competed off with excess free ATP, indicating a specific interaction with the nucleotide. Furthermore, fluorescent peptide binding assays revealed the affinity of 6TM-TAP (K_D 16 ± 15 nmol/L)

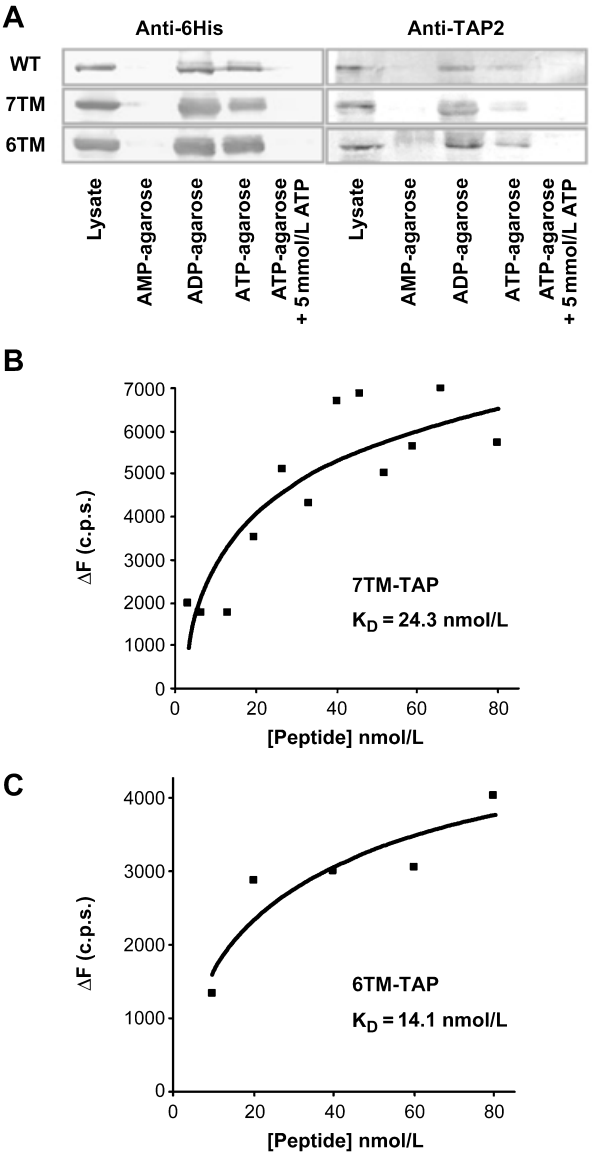

Figure 2 Deletion of the TAP N-terminal extensions did not destroy substrate binding. (A) Nucleotide binding properties of the three TAP constructs (WT-, 7TM- and 6TM-TAP). Membrane proteins solubilized from Sf21 cells harvested 48 h post-infection were incubated with nucleotide-agarose beads. The beads were washed, resuspended in SDS–PAGE running buffer and analysed by western blot with antibodies recognizing TAP1 (anti-6His) or TAP2 (anti-TAP2). WT-, 7TM- and 6TM-TAP all bound similarly to AMP-, ADP- and ATP-agarose beads and were all competed off with 5 mmol/L free ATP. Shown are representative blots from one of four separate experiments. (B) Peptide binding affinity was measured by fluorescence quenching of a fluorescein-labelled RRYQKCTEL peptide. Shown is a representative plot and its corresponding K_D constant. Average peptide affinity of 7TM-TAP from three experiments was $K_D = 21 \pm 11$ nmol/L. (C) Shown is a representative plot and its corresponding K_D constant. Average peptide affinity of 6TM-TAP from four experiments was $K_D = 16 \pm 15$ nmol/L.

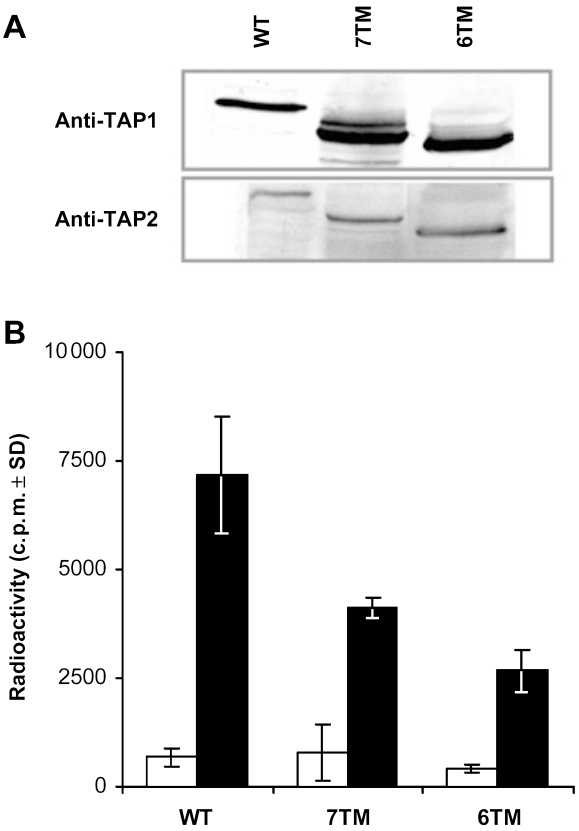

Figure 3 *In vitro* peptide transport activity of WT-, 7TM- and 6TM-TAP. Microsomes were isolated form baculovirus-infected Sf21 cells expressing the three TAP constructs. (A) Expression of WT-, 7TM- and 6TM-TAP was consistent in the three microsome preparations, as determined by western blot analysis. (B) *In vitro* peptide translocation activity. Microsomes were incubated with ^{125}I-labelled RRYNASTEL containing a glycosylation motif to prevent export. The microsomes were washed and radioactivity measured. Transport activity was stimulated by addition of 5 mmol/L ATP during incubation and the bar graph shows the results from one of two experiments, each performed in triplicate (□, –ATP; ■, +ATP). All three TAP constructs exhibited ATP-dependent transport activity.

and 7TM-TAP (K_D 21 ± 11 nmol/L) for the labelled RRYQKC-TEL peptide was comparable to the published dissociation constant for WT-TAP (19 ± 5 nmol/L)[17] (Fig. 2B,C). Hence, deletion of the N-terminal TM helices did not prevent TAP binding its nucleotide or peptide substrates.

7TM- and 6TM-TAP transport peptides in vitro

The truncated TAP variants transported a labelled RRYNAS-TEL peptide *in vitro*, although with reduced efficiency (Fig. 3). 7TM- and 6TM-TAP pumped peptide into Sf21-derived microsomes at a rate twofold and threefold lower than WT-TAP, respectively. However, in all three cases, ATP-dependent accumulation of radioactive peptides in the prepared microsomes was clearly observed. We conclude that ATP-dependent transport activity was preserved in 7TM- and 6TM-TAP, albeit at a lower rate.

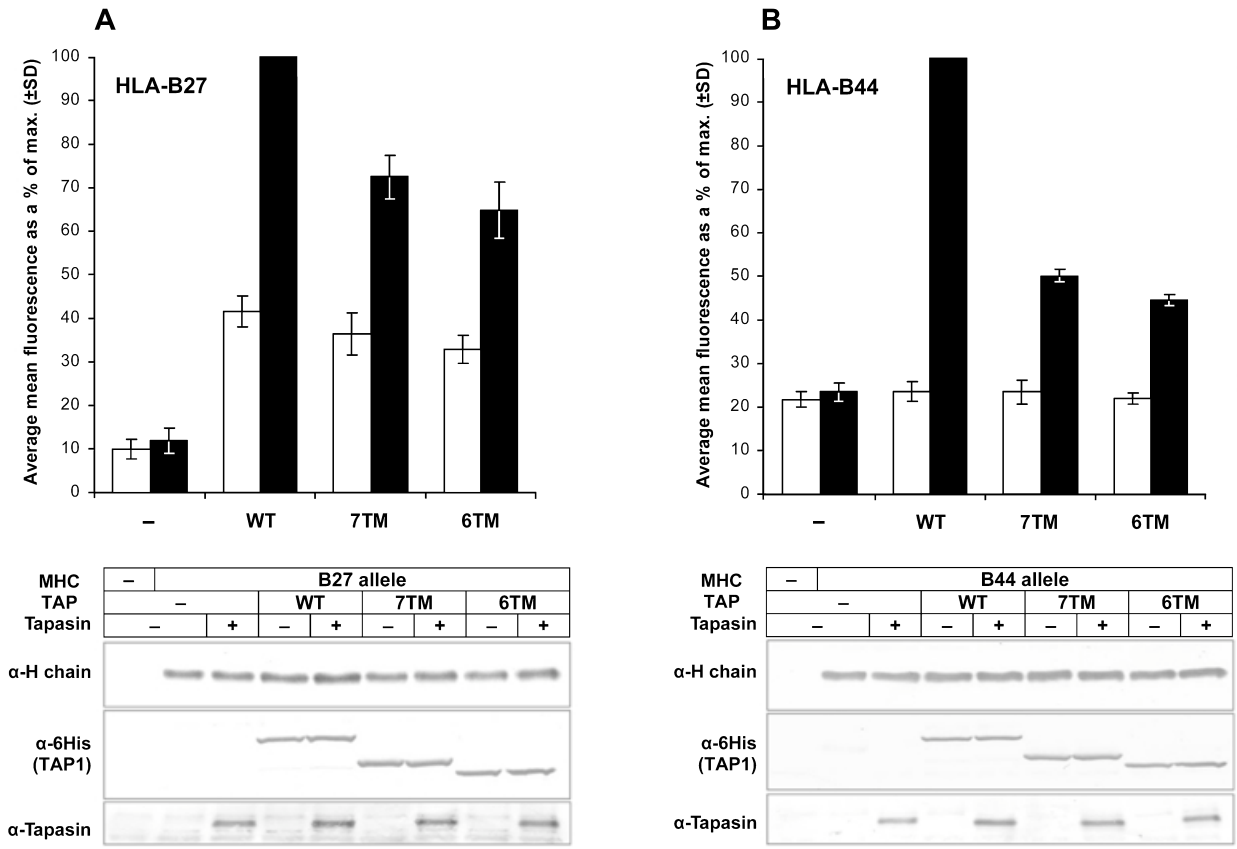

Figure 4 7TM-TAP and 6TM-TAP reconstitute antigen peptide loading onto MHC class I in insect cells. (A) Sf21 cells were co-infected with HLA-B27/β_2-microglobulin (multiplicity of infection; m.o.i. = 4), a TAP construct (m.o.i. = 8) and tapasin (m.o.i. = 4). So that all cells were triple infected, empty vector virus was used as a negative control for cells expressing only one or two of these three proteins. After 48 h, cells were harvested, stained with the anti-class I W6/32-FITC conjugate and analysed by flow cytometry. Mean fluorescence was compared to the HLA-B27/WT-TAP/tapasin sample (set at 100%) as an internal reference. Shown are the average results ± standard deviation from three separate experiments. □, Vector; ■, tapasin. Below the graph are western blots of infected cells from one representative experiment, indicating expression levels were consistent between samples. (B) As for (A), except that Sf21 cells were infected with HLA-B44 rather than HLA-B27.

7TM- and 6TM-TAP transport peptides in insect cells

To further analyse the transport activity of 7TM- and 6TM-TAP, they were co-expressed with β_2-microglobulin and the HLA-B27 class I protein in insect cells (Fig. 4A). As previously described, empty MHC class I is retained in the ER of insect cells, whereas peptide-loaded MHC class I molecules escape the ER and decorate the cell surface.[19] The detection of surface MHC class I on insect cells is hence an indicator of class I loading. For loading of peptides onto HLA-B27, only TAP co-expression is further required in insect cells. Therefore, the level of surface HLA-B27, measured by flow cytometry, provides an indirect measure of TAP activity within the cells. In this cell-based assay, WT-, 7TM- and 6TM-TAP were found to have equal transport activity, within error (Fig. 4A; □), verifying that deletion of the N-termini did not destroy basal transport activity. However, when the insect cells were co-infected with a tapasin-encoding virus, WT-TAP significantly out-performed 7TM- and 6TM-TAP (Fig. 4A; ■), indicating that deletion of the N-terminal segments diminished MHC class I loading in the presence of tapasin. The differences in MHC class I cell surface expression are consistently reproducible with small errors (see error bars in Fig. 4). Our signal strengths are similar to those of others who have used the same assay.[19]

To further investigate MHC class I loading in the presence of tapasin, we infected insect cells with a second class I protein, HLA-B*4402 (Fig. 4B). Peptide loading of HLA-B*4402 has previously been shown to be more dependent on tapasin than HLA-B27 in murine and human cells.[20] In our insect cell-based assay, surface expression of HLA-B44 was strictly dependent on tapasin, and only when cells were co-infected with tapasin did surface HLA-B44 levels rise above the control. Similar to HLA-B27, the loading and surface expression of HLA-B44 in the presence of tapasin was significantly greater with WT-TAP, whereas truncated 7TM- and 6TM-TAP yielded lower levels of surface HLA-B44.

Deletion of the TAP N-termini reduced co-precipitation with MHC class I and tapasin

The above results demonstrate that deletion of the TAP N-terminal segments severely impaired the action of tapasin. We hypothesized that 7TM- and 6TM-TAP may no longer

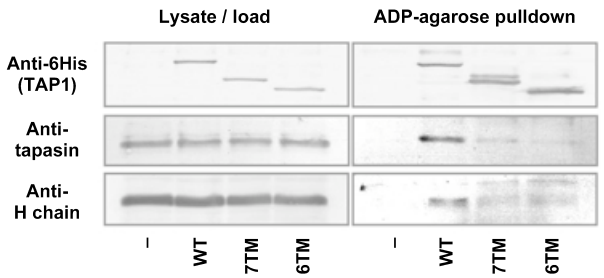

Figure 5 Tapasin and MHC class I co-precipitated with WT-TAP, but poorly associated with 7TM- and 6TM-TAP. Sf21 insect cells co-expressing a 6His-tagged TAP construct, tapasin and HLA-B27 were solubilized in 1% digitonin, and the TAP proteins precipitated with ADP-agarose beads. The beads were resuspended in SDS–PAGE loading dye, and analysed by gel electrophoresis and western blot. Shown are representative blots from three separate experiments.

interact physically with tapasin. To test this hypothesis, insect cells were co-infected with viruses encoding HLA-B27, tapasin and TAP. ADP-agarose was then used to precipitate TAP complexes from digitonin-solubilized membranes and the presence of tapasin or MHC class I in the TAP complex was assessed by western blot. Both tapasin and MHC class I were detected in WT-TAP precipitates, but both were nearly absent in 7TM- and 6TM-TAP precipitates (Fig. 5). Co-precipitation of tapasin with an anti-his antibody was also reduced in co-infections with 6TM-TAP/tapasin and 7TM-TAP/tapasin relative to WT-TAP/tapasin (data not shown). Compared to WT-TAP, significantly higher levels of 7TM- and 6TM-TAP expression were required to observe complexes with tapasin. The N-termini of TAP are hence required during formation of MHC class I peptide loading complexes.

Discussion

The experiments presented here describe the functional characterization of two TAP deletion variants. These variants are missing N-terminal accessory domains identified by protease-sensitive sites and sequence alignments to other ABC transporters. Based on our results, we conclude that the TAP subunits consist of an N-terminal accessory domain required for binding tapasin, a central 'pore' or TMD, and a C-terminal NBD (Fig. 1B). Furthermore, the TAP–tapasin interaction was found to be functionally significant for MHC class I loading.

First, deletion of the N-terminal accessory domain did not prevent binding to nucleotides or peptides. Nucleotides are bound by the cytosolic NBD,[21] and the peptide-binding site has been mapped to the last of the TMD cytosolic loops and the linker connecting to the NBD.[22] The N-terminal deletions in 6TM- or 7TM-TAP do not affect the previously identified substrate binding sites, and accordingly, the deletions did not impact nucleotide or peptide substrate binding (Fig. 2). This suggests that neither N-terminal deletion led to severe folding or structural abnormalities of the TAP transporter. The 6TM-TAP 'core transporter' has since been purified from infected insect cells by detergent extraction and chromatography, and is both functional (implying correct folding) and stable in detergent solution (E Procko and R Gaudet, unpubl. data, 2005). Functional differences between the TAP constructs are therefore relevant and not a by-product of reduced protein stability or misfolding.

Second, *in vitro* peptide transport activity was not abolished by deletion of the N-terminal accessory domains. Hence the two six-helix TMD and two cytosolic NBD conserved throughout the ABC transporter family are sufficient for TAP peptide transport activity. This is consistent with other ABC transporters, including MsbA and P-glycoprotein, which only feature these four domains and lack additional accessory sequences. According to our *in vitro* peptide transport assay data (Fig. 3), WT-TAP did possess higher transport activity than 6TM- and 7TM-TAP. This is perhaps because removal of the accessory domains alters the protein–phospholipid interface, which might affect the energetics of peptide transport. However, in the insect cell-based assay of peptide loading and cell surface localization of HLA-B27, the three proteins (WT-, 7TM- and 6TM-TAP) performed similarly (Fig. 4), indicating that peptide transport is not the rate-limiting step in this cell-based assay. The *in vitro* peptide transport assay and the cell-based assay measure slightly different properties. The insect cell assay quantifies the degree of MHC class I loading, which will depend on TAP activity, the shedding of ER-retaining chaperones, and the rate and stability of the class I peptide interaction. Perhaps due to these multiple events and/or to the fact that TAP is expressed at high levels in our insect cell expression system, the insect cell assay seems to have reduced sensitivity to discriminate between the different TAP peptide transport activity levels detected *in vitro*.

The third component of our data concerns the MHC class I-specific chaperone tapasin. Tapasin has been shown to associate with both MHC class I and TAP, linking the proteins together to form a peptide loading machine.[7,16] Tapasin also seems to stabilize TAP as it increases the steady-state levels of TAP protein in cells co-expressing TAP and tapasin.[10,11] However, class I surface expression could be fully restored in the tapasin-deficient .220 cell line by transfection of a soluble tapasin variant that had lost its ability to bind TAP.[23] Experiments where peptides were introduced into permeabilized microsomes in the absence of TAP also suggested that the tapasin–TAP interaction was unnecessary.[24] However, this was challenged in a recent report that only wild-type tapasin capable of binding TAP could fully rescue the tapasin-deficient .220.B*4402 cell line.[25] The significance of the tapasin–TAP interaction has hence remained unclear. Here, the N-terminal accessory domains of TAP were found to be necessary for higher order assembly of peptide loading complexes, with the simplest explanation being that these regions directly bind tapasin. During the preparation of this manuscript, Tampé and colleagues published results showing that deletion of the TAP N-terminus diminishes tapasin binding.[6] The current experiments further demonstrate the functional significance of this deletion on the class I loading pathway. Tapasin dramatically increased cell surface localization of MHC class I when co-expressed with WT-TAP in insect cells. However, deletion of the TAP N-terminus, which diminished the tapasin–TAP interaction, drastically reduced the effect of tapasin. This was particularly striking for the HLA-B44 (B*4402) allele, and correlates with the earlier

findings in .220.B*4402 cells.[25] Whereas in that study removing the C-terminal TAP-interacting region of tapasin impaired cell surface expression of HLA-B44, the corollary from the TAP perspective is illustrated here: removing the N-terminal tapasin-interacting accessory domain of TAP also impairs cell surface expression of HLA-B44. The residual enhancement observed with tapasin in 7TM- and 6TM-TAP-expressing cells is presumably from the tapasin–MHC interaction, which is expected to persist. The 7TM- and 6TM-TAP constructs described here offer useful tools for probing the significance of the TAP–tapasin interaction in other experimental systems.

The structures of the N-terminal accessory domains are currently unclear, although a strong hydrophobic character indicates a TM topology. Analysis of C-terminal deletion constructs in COS-7 cells supported eight and seven TM helices for full-length TAP1 and TAP2, respectively.[4] Others have predicted 10 and nine TM helices.[6] However, due to high identity between TAP1 and TAP2 (38%), the two subunits are thought to have arisen from an ancestral homodimer following gene duplication. It would therefore seem likely that both proteins have the same number of TM helices, as shown in the figures here. Individually expressed full-length TAP1 and TAP2 subunits also both bind tapasin,[26] which together with the present data suggests the N-terminal accessory domains share a common function and perhaps structure. Short of an X-ray crystal structure, this question might be further investigated by construction of TAP1–TAP2 fusions in a range of combinations. This would clarify which of the protein termini occupy the same side of the ER membrane, as such fusions containing a suitable linker would be predicted to produce a competent transporter.

Two X-ray crystal structures of MsbA, a bacterial multidrug ABC transporter homologous to TAP, have now been reported.[27,28] These two structures present distinct conformations and in both cases the cytosolic N-termini of each subunit in the dimer are facing outwards – away from the dimerization interface of the transporter. Sequence analyses show a significant similarity between MsbA and 6TM-TAP, leading to the expectation of a similar structural organization for 6TM-TAP. The N-terminal accessory domains of TAP1 and TAP2 would then extend out on opposite sides of the core transporter, as has been previously suggested.[6] The alternative, that the N-terminal domains are adjacent on the same side of the dimer, would imply something similar to a mirror line separating the homologous TAP1 and TAP2 subunits, a most unexpected result in a chiral protein. Instead, we favour a pseudo-twofold rotation axis. Stoichiometric analysis has revealed that each class I loading complex has four MHC and four tapasin molecules bound to a single TAP, with other housekeeping chaperones bound in substoichiometric quantities.[16] Based on these numbers, an arrangement where two tapasin and two MHC molecules are clustered around each of the TAP accessory domains is hypothesized (Fig. 6). Recent cross-linking experiments found no evidence that tapasin forms higher order multimers, suggesting four independent tapasin-binding sites on TAP,[29] supporting the model illustrated in Figure 6. The TAP accessory domains would then be packed against the conserved TMD, such that motion of one might alter function of the other. In this way, tapasin binding to the accessory domain could alter TAP transport activity, and TMD motions associated with peptide translocation

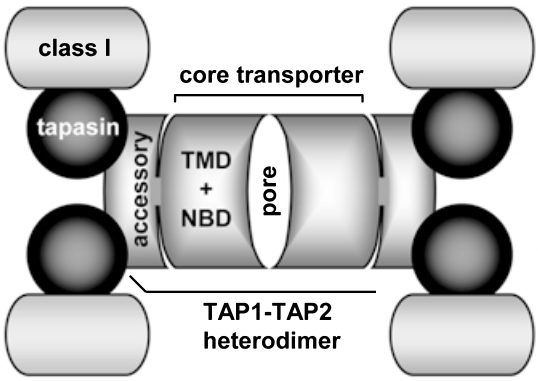

Figure 6 Possible structural organization of the peptide loading complex as viewed from the endoplasmic reticulum (ER) lumen. The transmembrane domains (TMD) from TAP1 and TAP2 line the 'pore' through which peptide is translocated, while the TAP N-terminal accessory domains lie adjacent in the plane of the ER membrane. Considering stoichiometric analysis data, two tapasin and two MHC class I molecules are proposed to associate with each N-terminal accessory domain. Conformational changes may be transmitted from TAP to tapasin to MHC class I, or vice versa, through the N-terminal accessory domains. NBD, nucleotide binding domain.

might communicate in the opposite direction back down the assembly line. In support of this, it has been noted that TAP conformation is coupled to MHC class I release.[30] Simple TAP–MHC class I tethering is unlikely to be tapasin's only role, although it may nevertheless be an important function. As a final comment, the protease sensitivity of lumenal loops in the N-terminal accessory domains suggests flexible, exposed residues in this region. If true, these flexible lumenal loops may support motions of the N-terminal TM helices, providing a mechanism by which tapasin binding to the N-terminus could modulate TAP structure and activity. More detailed biochemical and structural studies will be needed to answer these questions and hypotheses.

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