Exploring the minimal functional unit of the transporter associated with antigen processing

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Abstract TAP, an ABC transporter in the ER membrane, provides antigenic peptides derived from proteasomal degradation to MHC class I molecules for inspection by cytotoxic T lymphocytes at the cell surface so as to trace malignant or infected cells. To investigate the minimal number of transmembrane segments (TMs) required for assembly of the TAP complex based on hydrophobicity algorithms and alignments with other ABC transporters we generated N-terminal truncation variants of human TAP1 and TAP2. As a result, a 6 + 6 TM core-TAP complex represents the minimal functional unit of the transporter, which is essential and sufficient for heterodimer assembly, peptide binding, and peptide translocation into the ER. The TM1 of both, core-TAP1 and core-TAP2 are critical for heterodimerization of the complex.

Keywords: ABC-transporter; Antigen processing; MHC class I; Peptide-loading complex; Peptide transport

1. Introduction

The survival of vertebrates eminently depends on the adaptive immune system to protect the organism against invaders or cancer. For this purpose, the MHC class I-dependent pathway of antigen processing can trigger elimination of affected cells by cytotoxic T lymphocytes (CTL) upon presentation of antigenic peptides at the cell surface. The majority of peptides are derived from proteasomal degradation [1] and, for this purpose, the MHC class I-dependent pathway of antigen processing (TAP) [3]. After loading MHC class I molecules with high-affinity peptides, peptide-MHC complexes are shuttled to the plasma membrane for inspection by CTL [3–5].

2. Materials and methods

2.1. Cloning and expression of TAP constructs

To generate deletion mutants of human TAP1 (miniSTAP1, Δ2–211) and TAP2 (miniSTAP2, Δ2–174), the primer pairs 5'-CAGCTCGAGATGACTGACTGGATTCTACAAGATG-3', and 5'-CTGCTCGAGTCATTCTGGAGCATCTGCAGGAGCC-3', and 5'-CTGGCGGCCGCTCAGAGCTGGGCAAGCTTCTGC-3' were used, respectively. Different combinations of TAP1 and TAP2 (miniSTAP1/miniSTAP2, miniSTAP1/miniSTAP2, miniSTAP1/miniSTAP1, miniSTAP2/miniSTAP2) were cloned into the expression vector pFASTBac™ Dual (Invitrogen). Recombinant baculoviruses were generated using the Bac-to-Bac© Baculovirus Expression system according to the manufacturer’s instructions (Invitrogen).

2.2. Cell culture and microsome preparation

Insect cells (Spodoptera frugiperda (Sf9)) were grown in SF900II medium (Invitrogen) following standard procedures. Infection with recombinant baculovirus and preparation of microsomes were performed as described [15].

2.3. Peptide-transport assays

2.5 × 10^6 Sf9 cells were incubated with 0.01% of saponin in a total volume of 50 μl AP-buffer (5 mM MgCl₂ in PBS) for 1 min at 27 °C. Cells were washed twice with AP-buffer to remove the excess of detergent. Transport was performed with 500 nM of fluorescein-labeled peptides.
After double washing of the beads with lysis buffer, bound peptides were co-immunoprecipitated on magneto beads (Dynabeads/C210·antibodies) were visualized by chemiluminiscence imaging. TAP1- and TAP2-specific mAbs 148.3 and 435.3, respectively. Bound cells were analyzed by SDS–PAGE and immunoblotting with the human TAP1- and TAP2-specific mAbs 148.3 and 435.3, respectively. Bound antibodies were visualized by chemiluminescence imaging. peptide (RRYONST9L; N-core glycosylation site is underlined and ϕ indicates fluorescein coupled via a cysteine residue) in the presence of 10 mM ATP or 1 U of apyrase for 3 min at 32 °C. Peptide transport was stopped with 1 ml of AP-buffer supplemented with 10 mM EDTA. After centrifugation at 13000 × g, membranes were solubilized in 1 ml of lysis buffer (50 mM Tris/HCl, 150 mM NaCl, 5 mM CaCl2, 1 mM MnCl2, 1% NP-40; pH 7.5) for 10 min at 27 °C. Insoluble proteins were removed by centrifugation at 20000 × g and glycosylated peptides were bound to concanavalin A Sepharose overnight at 4 °C. After double washing of the beads with lysis buffer, bound peptides were eluted with 200 mM of methyl-α-D-mannopyranoside in lysis buffer for 30 min at 27 °C and quantified in a 96-well plate reader (Fluorostar Galaxy, BMG Labtechnologies, Jena, Germany).

2.4. Peptide-binding assays
Microsomes (100 μg protein) were incubated with 2 μM of the fluorescein-labeled RRYQKSTEL peptide in the absence or presence of a 400-fold excess of non-labeled competitor peptide (RRYQKSTEL) in 100 μl of AP-buffer for 15 min on ice. After solubilization with 1% SDS in PBS, membrane associated peptides were quantified in a 96-well plate reader. Peptides specifically bound to TAP were determined as the amount of labeled peptide, which was displaced from the peptide-binding pocket.

2.5. Co-immunoprecipitation
Microsomes (750 μg protein) were solubilized in 1 ml of lysis buffer (50 mM Tris/HCl, 150 mM NaCl, 5 mM MgCl2, 1% digitonin; pH 7.5) for 60 min on ice. Insoluble proteins were removed by centrifugation at 100000 × g for 45 min at 4 °C. Solubilized TAP1 and TAP2 were co-immunoprecipitated on magneto beads (Dynabeads® M-280 sheep anti-mouse IgG, Dynal Biotech), which were loaded with the TAP2-specific mouse monoclonal antibody 435.3[15] prior to incubation with solubilized protein. After incubation for 2 h at 4 °C, the beads were washed three times with 1 ml of washing buffer (50 mM Tris/HCl, 150 mM NaCl, 2 mM EDTA, 0.01% digitonin; pH 7.5). Proteins associated with the beads were eluted with 40 μl of SDS sample buffer containing 300 mM DTT and subsequently analyzed by SDS–PAGE and immunoblotting with the TAP1-specific rabbit antiserum lp2. The polyclonal antibody lp2 was raised against the peptide epitope ETEFFQQNQTGNIMSR according to Nijenhuis and Hämmerling[16].

3. Results and discussion
The TMD of most ABC transporters comprises six transmembrane segments (TM); however, extra N-terminal extensions have evolved, which are related to secondary functions. As for TAP, the 6 + 6 TM core complex (annotation of helices based on hydrophobicity analysis and alignment with other ABC transporters of subfamily B) is sufficient to allow for peptide binding and transport, whereas the N-terminal domains are important for assembly of the peptide-loading complex via recruitment of the adapter protein tapasin[12]. A comparison of different structures of membrane transporters made it evident that 7–10 TMs are required to form a transport pathway[17]. Proteins with less TMs need oligomerization to pattern a tunnel through the membrane. On the basis of these observations, we investigated a further possible subdivision of the core-domains of TAP1 and TAP2.

As truncation mutants of human TAP1 and TAP2 proved useful to examine the structural organization of the peptide-loading complex and the structure–function relationship of the heterodimeric complex[12], several deletion mutants of TAP1 and TAP2 with five or six remaining TMs were generated (mini5/STAP (Δ2–211/Δ2–174), mini6/STAP (Δ2–211/Δ2–122), and mini6/STAP (Δ2–166/Δ2–174)) (Fig. 1A). These TAP variants were heterologously expressed in insect cells (Sf9) from a single baculovirus encoding both TAP1 (p10 promoter) and TAP2 (polyhedrin promoter) as shown by immunoblotting using human TAP1- or TAP2-specific mouse monoclonal antibodies (Fig. 1B).

To elucidate whether peptide transport was preserved in these truncated TAP complexes we analyzed TAP-specific peptide transport in semi-permeabilized cells. Therefore, Sf9 cells expressing various TAP constructs were permeabilized with saponin and used in peptide translocation assays with a fluorescein-labeled peptide (RRYQNST9L), containing an N-core glycosylation site to monitor its translocation into the ER-lumen. Surprisingly, although nearly equal amounts of the TAP constructs were expressed as shown by immunoblotting with TAP1- and TAP2-specific mouse monoclonal antibodies (inlet
Fig. 2), all of the TAP variants were deficient in peptide transport when compared to wt TAP (Fig. 2). Similar results were obtained using isolated microsomes and radiolabeled peptide (RRYQNSTEL) in an experimental setup as described previously (data not shown; [12]). Note, mini6/6TAP shows the same transport activity as wt TAP [12].

Since TAP-dependent peptide translocation into the ER is a dynamic process, which requires multiple structural rearrangements within the TMDs and the NBDs [3], we next analyzed peptide binding of the TAP constructs, representing the initial step in peptide transport. Isolated microsomes were incubated with 2 µM of fluorescein-labeled peptide (RRYQSTEL; $K_d = 93 \pm 9$ nM [18]) in saturation binding experiments. The extent of TAP-specific peptide binding was determined as the amount of fluorescent peptide, which could be competed from the binding pocket of the complex with an excess of non-labeled peptide (RRYQSTEL; $K_d = 213 \pm 21$ nM [12]). Both, mini5/6TAP and mini6/5TAP showed less than 20% of specific peptide binding activity when compared to wt TAP (Fig. 3). Mini6/6TAP has the same peptide binding affinity as wt TAP [12]. However, no binding above background was detected for mini5/5TAP (Fig. 3). The peptide binding pocket is localized in the cytosolic loops between TM4 and TM5 and a stretch of 15 amino acids C-terminal of TM6 of the core domains of TAP1 and TAP2 [16]. All of these regions are present in the TAP constructs displayed in our study.

Both, TAP1 and TAP2 contribute to peptide binding [19], indicating that heterodimerization is a prerequisite for peptide binding. Therefore, impaired activity of the truncation variants could be due to a deficiency in heterodimer formation. We therefore performed co-immunoprecipitation experiments with a TAP2-specific antibody. Surprisingly, mini6/6TAP and mini6/5TAP formed heterodimers as shown by immunoblotting with a TAP1-specific rabbit polyclonal antibody, whereas mini5/5TAP did not (Fig. 4). Worth mentioning, an additional non-identified band of about 72 kDa was detected in the detergent-solubilized fraction of mini5/5TAP. Our data clearly demonstrate that impaired peptide transport and peptide binding of mini5/6TAP and mini6/5TAP are not caused by a loss of the assembly of the heterodimeric complex but due to a disturbance of dynamic processes within the heterodimer.

On sequence level the TMs in the core-TAP complex align well with MsbA and P-glycoprotein, of which the X-ray and low resolution structure were solved, respectively [20,21]. Although it is a matter of debate whether the structure of the MsbA homodimer does indeed reflect the native domain interface, also because the dimer interface would be too small to confer thermodynamic stability [20,22], the packing of the TMs of the MsbA monomer is similar to that observed for P-glycoprotein [20]. Here, we provide biochemical evidence that for the core-TAP complex also the TM1 are needed to form a stable heterodimer interface.

Taken together, our data point out that the core-TAP complex is essential and sufficient for heterodimerization of the TAP complex, peptide binding and transport with the TM1 of core-TAP1 and core-TAP2 being crucial for heterodimerization. However, it remains unclear how these TMs contribute to the formation of an active transport complex. In spite of the existence of ABC transporters with less than 6 TMs, such as the E. coli macrolide antibiotic exporter MacB [13] and the lipoprotein exporter LolC/LolE [14], respectively, our data moreover our data show clearly that a 6 + 6 TM core-domain represents the minimal active transporter for human TAP.

Further structural characterization of the TMDs of the TAP heterodimer and related ABC transporters, especially with respect to the relative orientation of the TMs to one another at the dimer interface, will be essential for the understanding of how the substrate translocation pathway is formed.

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