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Impaired Transporter Associated with Antigen Processing (TAP) Function Attributable to a Single Amino Acid Alteration in the Peptide TAP Subunit TAP1[1]

Ulrike Ritz,* Ingo Drexler,† Dirk Sutter,‡ Rupert Abele,§ Christoph Huber,* and Barbara Seliger[2]*

The heterodimeric peptide transporter TAP belongs to the ABC transporter family. Sequence comparisons with the P-glycoprotein and cystic fibrosis transmembrane conductance regulator and the functional properties of selective amino acids in these ABC transporters postulated that the glutamic acid at position 263 and the phenylalanine at position 265 of the TAP1 subunit could affect peptide transporter function. To define the role of both amino acids, TAP1 mutants containing a deletion or a substitution to alanine at position 263 or 265 were generated and stably expressed in murine and human TAP1$^{-/-}$ cells. The different TAP1 mutants were characterized in terms of expression and function of TAP, MHC class I surface expression, immune recognition, and species-specific differences. The phenotype of murine and human cells expressing human TAP1 mutants with a deletion or substitution of Glu^{263} was comparable to that of TAP1$^{-/-}$ cells. In contrast, murine and human TAP1 mutant cells containing a deletion or mutation of Phe^{265} of the TAP1 subunit exhibit wild-type TAP function. This was associated with high levels of MHC class I surface expression and recognition by specific CTL, which was comparable to that of wild-type TAP1-transfected control cells. Thus, biochemical and functional evidence is presented that the Glu^{263} of the TAP1 protein, but not the Phe^{265}, is critical for proper TAP function. ***The Journal of Immunology*****, 2003, 170: 941–946.**

The TAP belongs to the large family of ABC transporters and shares the typical three-domain structure of these transporters. TAP consists of a C terminus spanning the membrane of the endoplasmic reticulum (ER)[3] several times, a substrate binding domain, and the hydrophilic nucleotide binding domain containing the Walker A and B consensus motifs (1). ABC transporters translocate a variety of substances/molecules, including ions, sugars, amino acids, peptides, and polysaccharides, but each transporter is specialized for the transport of one unique substrate or class. The best characterized human ABC transporters are the cystic fibrosis transmembrane conductance regulator (CFTR), a chloride channel that is defective in cystic fibrosis (2), and the P-gp (3) that export hydrophobic drugs, lipids, and phospholipids out of the cell.

In the P-gp as well as in the CFTR protein, point mutations causing an amino acid substitution or deletion have been identified, which result in either a loss of function or alterations in quality and quantity of substrate transport: in CFTR point mutations of the amino acids, Phe^{508}, Tyr^{109}, Pro^{208}, and Glu^{92} close the channel (4, 5), whereas mutations at His^{117}, Phe^{334,337}, and Arg^{347} result in a lower chloride flow rate through the channel (6). Substitutions of Phe$^{72, 335, 522–525, \text{and } 978}$ to Ala of the P-gp change the transport specificity, which can be associated with a distinct substrate binding and consequently with an altered drug-resistant profile of cells (7, 8).

Although TAP is a typical ABC transporter with the highest sequence homologies to P-gp, it differs in its structure and function from other transporters of this family. TAP is the only ABC transporter with a function in the immune system as it translocates antigenic peptides length and sequence specific from the cytosol into the ER, where they are loaded onto MHC class I molecules (9). Moreover, TAP consists of two subunits, TAP1 and TAP2, and the formation of a heterodimeric complex is required for its proper function (1, 10, 11).

It is apparent that TAP is involved in numerous interactions inside the ER and the cytosol, but the exact features of TAP have not yet been defined. Based on structural and functional analyses, different topology models for TAP have been postulated. Functional analyses of C-terminal deletion mutants as well as peptide cross-linking experiments to the human TAP defined multiple regions in both subunits, which putatively contribute to the peptide binding site (12, 13). Using a series of deletion mutants in the potential peptide binding domain, the residues 366–405 of the TAP1 subunit have been identified to contain structural and/or functional relevant sequences for peptide transport (14). Furthermore, many groups characterized the ATP binding domain of TAP using a panel of deletion or chimeric mutants of TAP1 and TAP2, thereby demonstrating an influence of the NTP binding on TAP function, conformational changes, and peptide binding (15–19).

To provide information about the structural features of TAP that alter its function, the TAP sequence was compared with that of P-gp and CFTR and their known residues/amino acids resulting in functional changes. Using this approach, 2 aa potentially important for TAP function, the glutamic acid at aa position 263 and the phenylalanine at aa residue 265, respectively, were postulated.

*Third Department of Internal Medicine, Johannes Gutenberg-University, Mainz, Germany; †GSF Research Center, Institute for Molecular Virology, Munich, Germany; ‡Technical University, Institute for Virology, Munich, Germany; and §Johann Wolfgang Goethe-University, Institute for Biochemistry, Frankfurt, Germany

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[2] Address correspondence and reprint requests to Dr. Barbara Seliger, Johannes Gutenberg University, Third Department of Internal Medicine, Langenbeckstr. 1, 55101 Mainz, Germany. E-mail address: B. Seliger@3-med.klinik.uni-mainz.de

[3] Abbreviations used in this paper: ER, endoplasmic reticulum; CFTR, cystic fibrosis transmembrane conductance regulator; wt, wild type.

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TAP1 mutants containing either a deletion or a substitution to alanine at Glu^{263} or Phe^{265} were subsequently characterized in terms of protein expression, ER localization, dimer formation with TAP2, peptide transport, MHC class I surface expression, and specific lysis by MHC class I-restricted CTL. By comparing the biochemical and functional properties of the cell lines expressing TAP1 mut/del 263 and TAP1 mut/del 265, we present evidence in this work that Phe^{265} of TAP1 has a minimal effect, whereas Glu^{263} of TAP1 has a striking effect on TAP function.

Materials and Methods

Construction of TAP mutants

The vector pBluescript SK^+ containing the human 2.3-kb TAP1A cDNA served as a template for standard PCR (1 min, 95°C; 1 min, 54°C; 1 min and 30 s, 72°C for 25 cycles) using the native *Pfu* polymerase (Stratagene, Amsterdam, The Netherlands) and TAP1-specific primers. The forward primers 1 contain a *Bbr*PI restriction site (italic letters) and the deletion or the desired mutation to alanine at aa position 263 or 265 (bold letters), respectively; the reverse primer 2 contains a *Spe*I restriction site (italic letters; Table I). The resulting amplification products of 500 or 503 bp were then reintroduced into their original positions of the TAP1 wild-type (wt) cDNA. Upon confirmation of the integrity of the substituted or deleted amino acid by sequencing, the designed TAP1 mutants were cloned into the *Eco*RV site of the expression vector pIRES-neo (20).

Cell lines and culture

The murine $TAP1^{-/-}$ cells kindly provided by H.-G. Ljunggren (Karolinska, Stockholm) were established from a methylcholanthrene-induced tumor of a $TAP1^{-/-}$ C57BL/6 (B6) mouse ($H\text{-}2^b$) (21); the human $TAP1^{-/-}$ BRE cells kindly provided by H. delaSalle were derived from a patient with bare lymphocyte syndrome (22). The $TAP1^{-/-}$ cells were either maintained in RPMI medium (mouse $TAP1^{-/-}$ cells; Seromed, Frickenhausen, Germany) or in DMEM (human BRE cells) supplemented with 10% FCS (Greiner, München, Germany), 2 mM L-glutamine, 100 μg streptomycin/ml, and 100 U penicillin/ml medium.

Stable transfection of $TAP1^{-/-}$ cells

Gene transfer of $TAP1^{-/-}$ cells was performed by lipofection using the wt or mutant TAP1 constructs and/or the HLA-A*0201 expression vector (23), as recently described (14). Stable neomycin-resistant (neo^R) transfectants were selected in culture medium supplemented with 500 μg/ml G418.

Immunofluorescence and flow cytometric analyses

For immunofluorescence, 1×10^5 cells/well were seeded on sterile cover slips or in six-well plates for 24 h, then washed twice with PBS, followed by fixation with 4% paraformaldehyde in PBS for 10 min. After blocking, the cells were incubated with the appropriate concentration of the human anti-TAP1 (24) or anti-calnexin (Affinity, Golden, U.K.) mAb, respectively. Cells were then incubated with Hoechst stain (Roche Biochemicals, Mannheim, Germany) and using as a secondary Ab a goat anti-mouse FITC-conjugated Ig for TAP1 and a goat anti-mouse Cy-3 for calnexin, respectively. Images were obtained using a confocal laser microscope (Leica, Buffalo, NY).

Indirect immunofluorescence analysis was performed by flow cytometry, as recently described (14). Briefly, 5×10^5 cells were incubated with the appropriate concentration of the primary Ab (anti-H2 K^bD^b; Cedarlane Laboratories, Hornby, Ontario, Canada) or HLA-ABC (Coulter/Beckman, Krefeld, Germany) for 30 min on ice, then washed twice with PBS and additionally incubated with a goat anti-mouse FITC Ig for 30 min at 4°C. A total of 10,000 viable cells were analyzed on a flow cytometer (Coulter Epics XL MCL software, Coulter Epics XL MCL System II 3.0; Coulter/Beckman). A mouse anti-IgG2a served as control, and the mean specific fluorescence intensity was calculated by setting the results of the control and the probe in relation.

Immunoprecipitation

A total of 2×10^6 cells were lysed with buffer containing 1% Nonidet P-40 before adding a murine anti-TAP2 mAb (kindly provided by K. Früh, San Diego, CA) overnight at 4°C, followed by the addition of protein A-Sepharose for 4 h. After two washing steps, the bound proteins were eluted, precipitated with TCA, and further subjected to Western blot analysis (see below).

ATP-agarose-binding assays

ATP-agarose-binding assays were performed, as previously described (15). A total of 1×10^7 cells were disrupted by freezing and thawing, and the resulting cell suspension was centrifuged at $1{,}000 \times g$ for 10 min at 4°C. The supernatant was spun at $100{,}000 \times g$ for 1 h. Membrane pellets were resuspended in lysis buffer (TBS/$MgCl_2$/2% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate), incubated for 30 min on ice before centrifuging at $10{,}000 \times g$ for 30 min. Proteins were incubated with ATP-agarose, eluted with elution buffer, and subjected to Western blot analysis.

Western blot analysis

For the detection of TAP proteins, samples from both the immunoprecipitation and the ATP-binding assays were size fractionated by 10% SDS-polyacrylamide gels, visualized by Ponceau S, and transferred onto nitrocellulose membranes (Schleicher & Schüell, Dassel, Germany). The membranes were blocked with TBS/10% horse serum/5% milk powder/0.1% Tween 20 for 2 h, briefly rinsed with TBS/0.1% Tween 20, and additionally incubated with the anti-human TAP1 mAb 148.3 recognizing the C terminus of TAP1 for 2 h. After rinsing twice in TBS/0.1% Tween 20, membranes were incubated with either alkaline phosphatase- or HRP-conjugated rabbit anti-mouse Ig-specific Ab (DAKO, Hamburg, Germany) for 1 h, followed by two washing steps. Then, the membranes were developed with the ECL determination kit (Amersham Pharmacia, Freiburg, Germany) or using nitroblue tetrazolium/X phosphate as substrate (Roche Biochemicals).

Membrane preparation and peptide-binding assays

Cells were thawed on ice in 10 mM Tris buffer (pH 8.0) supplemented with protease inhibitors and 1 mM DTT and homogenized with a glass homogenizer. Lysed cells were centrifuged at $1000 \times g$ for 10 min. For collection of crude membranes, supernatant was centrifuged at $200{,}000 \times g$ for 30 min and pellets were resuspended in PBS. The protein concentration of the crude membranes was determined by micro bicinchoninic acid protein assay. For peptide binding, the peptide RRYQKSTEL was labeled by the chloramine-T method using 1.5 nmol peptide and 100 μCi ^{125}I. To determine the amount of functional TAP, the membranes were incubated with radiolabeled peptide (200 nM) in a total volume of 50 μl in PBS, 5 mM $MgCl_2$ for 15 min on ice. Unbound peptide was removed by adding 500 μl PBS, 5 mM $MgCl_2$, and centrifuged for 8 min at $20{,}000 \times g$. The supernatant was discarded, and the washing procedure was repeated once. The membrane-associated radioactivity was directly quantified by gamma counting.

Table I. *Primers used for PCR mutagenesis*

	Primer 1, Position 754	Primer 2, Position 1257
Deletion of Glu^{263}	5′-accatgggc*cacgtg*cacagccacttgcaggga **DEL263**gtgtttggggctgtcctgcgccag-3′	
Mutation of Glu^{263} to Ala	5′-accatgggc*cacgtg*cacagccacttgcaggga **gca**gtgtttggggctgtcctgcgccag-3′	5′-acctgaaat*actagt*ggt ccagga-3′
Deletion of Phe^{265}	5′-accatgggc*cacgtg*cacagccacttgcaggga gaggtg**DEL265**ggggctgtcctgcgccag-3′	
Mutation of Phe^{263} to Ala	5′-accatgggc*cacgtg*cacagccacttgcaggga gaggtg**gca**gggctgtcctgcgccag-3′	

Peptide transport assay

Peptide translocation assays were conducted on streptolysin O-permeabilized cells using the reporter peptides TNKTRIDGQY and RRLYQNSTEL, as described by Neefjes et al. (25). Shortly, the peptides were radiolabeled with ^{125}I by the chloramine T method using 10 nmol peptide, 1.0 mCi ^{125}I, and Sephadex G-10 columns for removal of unincorporated iodine. After trypsinization, 2.5×10^6 TAP1$^{-/-}$ cells or transfectants were permeabilized with 2 IU streptolysin O (Welcome Reagent, Beckenham, U.K.) in 50 μl incubation buffer (130 mM KCl, 10 mM NaCl, 1 mM $CaCl_2$, 2 mM EGTA, 2 mM $MgCl_2$, and 5 mM HEPES, pH 7.3) for 5 min at 37°C, followed by the addition of 10 μl ATP (10 mM; Roche) and 2.5 μl of radioiodinated peptides (0.5 μM) in a final volume of 160 μl, and incubated at 37°C for 20 min. Subsequent to cell lysis in 1% Nonidet P-40 (Sigma-Aldrich, St. Louis, MO), glycosylated peptides were recovered by adding Con A-Sepharose beads (Pharmacia, Uppsala, Sweden) overnight, and after extensive washing the radioactivity associated with the beads was measured by gamma counting. Translocation efficiencies were calculated according to the formula: cpm (glycosylated labeled peptides bound to Con A beads)/cpm (total label of peptides) $\times$ 100.

Cytotoxicity assay of TAP1 transfectants

Human BRE$^{-/-}$ cells and their wt or mutTAP1/HLA-A2-transfected derivatives were used as targets for HLA-A2-restricted tyrosinase-specific CTL using the standard chromium release assay (26). Cells were infected with 10 U/cell of vaccinia-tyrosinase virus and incubated at 37°C, as described (27). After 3 h, cells were labeled with ^{51}Cr and further incubated at 37°C. Twelve hours postinfection, cytotoxicity assays were performed. ^{51}Cr-labeled targets were mixed with different concentrations of effectors (E:T ratio) in triplicates and incubated for 4 h at 37°C. Supernatants were then harvested, and radioactivity was counted in a gamma counter. Percent specific lysis was calculated as 100 $\times$ (experimental release $-$ spontaneous release)/(maximal release $-$ spontaneous release).

Results

Identification of potentially important amino acids of TAP by sequence comparison with other ABC transporters

Single amino acids have been described in the CFTR as well as in the P-gp, which influence their binding specificities and/or function. These include the Glu^{92} of CFTR and the Phe^{72} of P-gp. Using computer-based sequence analyses, an overlapping stretch of CFTR and P-gp sequences was identified in the TAP1 subunit, postulating a potential importance of these 2 aa: the Glu^{263} and the Phe^{265} (Fig. 1). To explore the role of Glu^{263} and Phe^{265} in TAP function, both amino acids were either deleted (TAP1 del263 and TAP1 del265) or substituted to alanine (TAP1 mut263 and TAP1 mut265) by PCR mutagenesis. Wt and mutant TAP1 constructs were stably expressed in both murine (TAP1$^{-/-}$ C57BL/6) and human TAP1-negative cells (BRE fibroblasts). Upon selection in G418, stable integration of wt and mutant TAP1 cDNA was confirmed in neo^R clones by genomic PCR using construct-specific primers (data not shown).

The different mutTAP1 proteins are localized in the ER membrane, bind ATP, and form complexes with TAP2

Expression of human wt and mutant TAP1 proteins in murine and human TAP1$^{-/-}$ transfectants was determined by Western blot analysis. Murine and human TAP1$^{-/-}$ cells served as controls. A similar protein expression pattern of human wt and mutant TAP1 proteins was detected in murine and human cell lines analyzed (data not shown). To define the biological properties, ER localization, ATP-binding ability, and heterodimer formation of the different TAP mutants and the wt TAP1 transfectants were evaluated. Immunostaining with the anti-TAP1 mAb 148.3 revealed ER localization of all mut/del TAP1 proteins, as representatively shown for the murine cells transfected with the human TAP1 del263 mutant, which was comparable to that of wt TAP1 and calnexin (Fig. 2, *a*, *c*, and *d*). As expected, no TAP1-specific staining was detected in TAP1$^{-/-}$ cells (Fig. 2*b*). As representatively shown for the human BRE TAP1$^{-/-}$ cells, this was also associated with lack of TAP1/TAP2 complex formation (Fig. 3*a*, *lane 1*) and deficient ATP binding (Fig. 3*b*, *lane 1*). In contrast, the human cells transfected with human wt TAP1 and the different mut/del TAP1 constructs possess the capability to form heterodimers with TAP2 and to bind ATP (Fig. 3, *a* and *b*). Thus, both the deletions and amino acid replacements of Glu^{263} or Phe^{265} of the TAP1 subunit seem not to influence the structure of the peptide transporter because a protein product of the expected size, ER localization, heterodimer formation with TAP2, and ATP binding were detected.

Deletion or mutation of the glutamic acid at aa position 263 results in loss of TAP function and MHC class I surface expression

Human BRE cells expressing TAP1 mutants as well as TAP1-negative and wt TAP1-transfected control cells were investigated for peptide binding to TAP. As representatively shown in Fig. 4, peptide binding was abolished in the TAP1-negative control cells and in the human cells expressing TAP1 mutants with a point mutation at Glu^{263}. In contrast, peptide binding was demonstrated

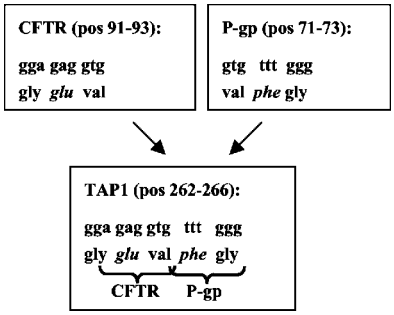


FIGURE 1. Sequence comparison of three ABC transporters. Sequences from two ABC transporters, CFTR and P-gp, which influence their function and/or binding specificity, were compared with that of the TAP1 subunit. Two amino acids potentially essential for proper TAP function, Glu^{263} and Phe^{265}, were identified and either deleted or mutated to Ala for further analyses.

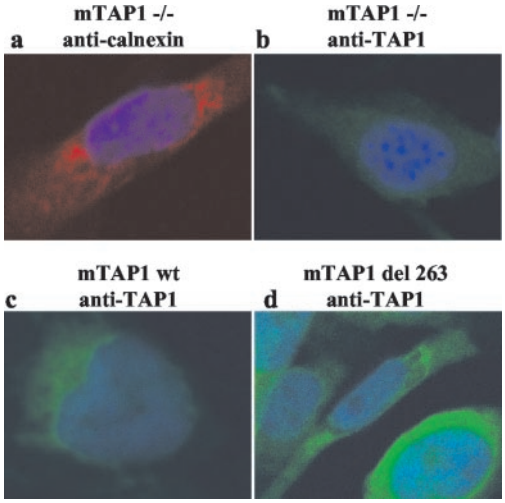


FIGURE 2. ER localization of mutTAP1 proteins. Immunfluorescence was performed as described in *Materials and Methods*. Calnexin (*a*) and wt TAP1 (*c*) served as positive controls for ER localization, whereas the TAP1$^{-/-}$ cells served as negative control (*b*). TAP1 del263 is clearly localized in the ER membrane at a position comparable to that of wt TAP1 and calnexin (*d*).

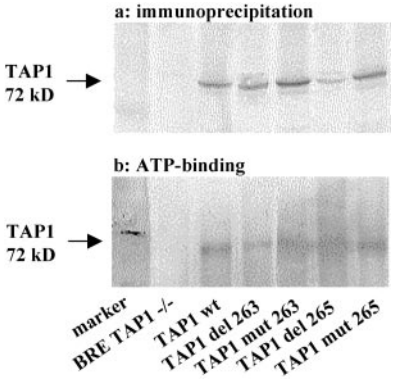


FIGURE 3. Heterodimer formation with TAP2 and ATP binding of the TAP1 mut/del proteins expressed in human BRE cells. Lysates of TAP1 mut/del transfectants and control cells were analyzed for the TAP complex formation by immunoprecipitation (*a*) and ATP-binding capacity (*b*), as described in *Materials and Methods*.

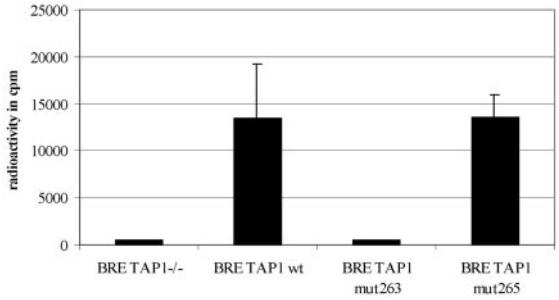


FIGURE 4. Peptide binding to wt TAP1 and TAP1 mut 263/265 transfectants. BRE cells were analyzed for peptide binding to TAP, as described in *Materials and Methods*. The results are expressed as recovered γ-cpm.

in the TAP1 mut265 cells (13,600 cpm), which was comparable to that of wt TAP1 BRE transfectants (13,400 cpm).

To confirm the different TAP function of TAP1 mut/del 263/265 cells, TAP-mediated peptide translocation into the ER was determined using two different iodinated reporter peptides. Murine NIH3T3 fibroblasts, human cervical carcinoma cells (HT3), wt transfectants, and TAP1$^{-/-}$ cells served as controls. As expected, murine and human cells transfected with human wt TAP1 transport peptides with a high efficiency of 2.9, 2.1, and 4.4%, respectively (Fig. 5*a*). In contrast, a heterogeneous peptide transport rate was demonstrated in the different mut/del TAP1 proteins: the human cells expressing human TAP1 mutants with either a deletion or point mutation at Glu^{263} lack peptide transport and show a transport efficiency similar to that of TAP1$^{-/-}$ cells (transport rate: 0.01–0.1%). In contrast, human cells containing TAP1 mutants with a deletion or point mutation at Phe^{265} were capable of translocating peptides with an efficiency ranging from 1.2 to 2.5%, depending on the peptide used. This efficiency was comparable to that of murine NIH3T3 fibroblasts and the cervical carcinoma cell line (HT3) exhibiting a peptide transport rate of 1.1 and 3.2%, respectively, but represents only 50% of the level of translocation of wt TAP1 transfectants.

Peptides translocated by TAP are loaded onto the β_2-microglobulin (β_2m)/MHC class I dimer, which is then transported to the cell surface and presented to CTL. TAP deficiencies result in the expression of unstable β_2-microglobulin/MHC class I dimers. To assess the functional consequences of the different peptide transport rate of TAP1 mut/del 263 and 265, MHC class I surface expression was analyzed by flow cytometry using MHC class I-specific Abs. As shown in Fig. 5*b*, the murine and human cell lines transfected with TAP1 mut/del 263 showed no MHC class I surface expression (Fig. 5*b*), whereas the TAP1 mut/del 265 cell lines expressed significant levels of MHC class I surface Ags that were comparable to that of wt TAP1 transfectants, murine NIH3T3, and human HT3 control cells.

Cells transfected with TAP1 del or mut 263 are not recognized by specific CTL

To define whether there exists a correlation between MHC class I surface expression and immune recognition, vaccinia tyrosinase-infected parental BRE TAP1$^{-/-}$ cells as well as BRE wt and TAP1 mut/del 263/265 variants were used as targets for HLA-A2-restricted tyrosinase-specific CTL. Before cytotoxicity assays, all cell lines were stably transfected with an HLA-A2 expression vector.

As expected, the HLA-A2-positive BRE TAP1 $^{-/-}$ cells were not lysed by tyrosinase-specific CTL. Similarly, the HLA-A2-expressing BRE TAP1 mut/del 263 cells were not recognized by these CTL. In contrast, BRE wt and TAP1 mut/del 265 transfectants as well as tyrosinase-transfected NA8 melanoma control cells were efficiently lysed (Fig. 6), thereby confirming the data from the peptide translocation assay and MHC class I surface expression.

Discussion

It has been postulated that the Glu^{263} and Phe^{265} of the TAP1 subunit are involved in the function of the peptide transporter. This assumption was based on sequence comparisons between TAP and two other members of the ABC transporter superfamily, the CFTR and P-gp, in which these amino acid residues alter the substrate specificity and function of the transporters, respectively (4, 7, 28, 29). The Glu^{263} and Phe^{265} of human TAP1 were either deleted or substituted to alanine, and the constructs were transfected into murine and human TAP1$^{-/-}$ cells. The TAP1 variants were analyzed regarding their individual contributions to TAP function. The experimental data concerning MHC class I surface expression of both the human and murine TAP1 del/mut-transfected cell lines were comparable. These data confirm the absence of species-specific differences between the human TAP1/TAP2 complex and the TAP heterodimer consisting of human TAP1 and murine TAP2, as described earlier (14, 30).

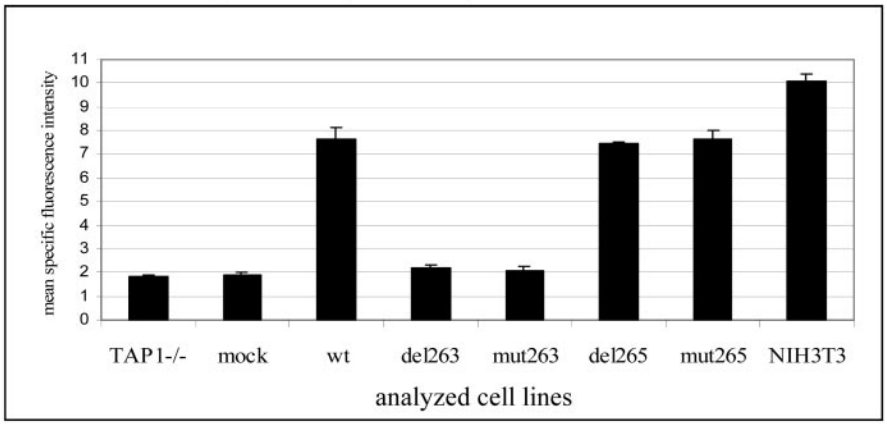
The deletion or mutation of aa 263 or 265 resulted in various effects on TAP function, despite their normal properties of TAP1 in terms of protein expression, ER localization, and ATP binding. These data suggest that the deletion or mutation of the aa residues 263 or 265 in TAP1 causes no conformational changes. However, a deletion or mutation to Ala at Glu^{263} of TAP1 causes a loss of transporter function, as measured by both peptide binding and translocation using model peptides, which have been demonstrated to be optimal substrates for TAP. This impaired TAP function was directly associated with the lack of MHC class I surface expression and CTL-mediated immune recognition. The peptide transport is proposed to be a two-step process that includes peptide binding, followed by the peptide transport cycle (19). Because alterations at Glu^{263} of TAP1 affect peptide binding, the mut 263 cells lack peptide transport. In contrast, a deletion or mutation to Ala in residue Phe^{265} of TAP1 does neither affect MHC class I surface expression nor immune recognition when compared with that of wt TAP1 transfectants or control cells. Concerning TAP function, peptide binding was not impaired in these cells, whereas a reduced transport activity of the TAP1 mut/del 265 transfectants in comparison with wt TAP1 control cells was detected with both peptides used. These data suggest that alteration at Phe^{265} of TAP1

a: peptide translocation assay

human cells (BRE TAP1 -/-)	TAP1 -/-	TAP1 wt	TAP1 del 263	TAP1 mut 263	TAP1 del 265	TAP1 mut 265	murTAP1 -/- wt	NIH3T 3	HT3
% transp. peptide (TNKTRIDGQY)	0,1 ± 0	*4,4 ± 0,3*	0,1 ± 0	0,1 ± 0	*2,5 ± 0,2*	*2,3 ± 0,3*	*2,9 ± 0,2*	*1,1 ± 0,1*	*3,2 ± 0,1*
% transp. peptide (RRLYQNSTEL)	0,01 ± 0	*2,1 ± 0,2*	0,01 ± 0	0,01 ± 0	*1,2 ± 0,1*	*1,2 ± 0,2*	**n.d.**	**n.d.**	**n.d**

b: MHC class I surface expression

murine TAP1 negative cells (mTAP1 -/-)



human TAP1 negative cells (BRE TAP1 -/-)

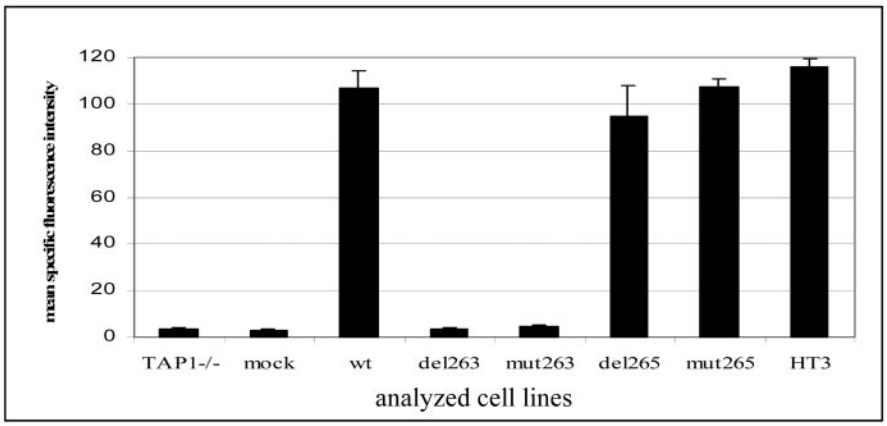


FIGURE 5. Peptide translocation and MHC class I surface expression of wt TAP1 and TAP1 del/mut-transfected TAP1$^{-/-}$ cells. A series of murine and human cells expressing human TAP1 del/mut 263/265 mutants and control cells (NIH3T3, HT3, and murTAP1$^{-/-}$) was analyzed for peptide transport utilizing the peptide translocation assay using two peptides (*a*) and MHC class I surface expression by flow cytometry (*b*), as described in *Materials and Methods*. The results are expressed as percentage of transported peptides (*a*) and as mean specific fluorescence intensity (*b*; MFI). All experiments were performed at least twice.

affects the peptide transport rather than peptide binding to TAP. This altered peptide transport specificity in comparison with wt TAP1 transfectants might be attributable to changes in the transport kinetic for these peptides. In this view, the amino acid Phe^{265} may play a role in the pore-forming activity of TAP and not as earlier proposed in peptide binding (12–14). Similar effects have been described for different amino acid substitutions in the P-gp (7).

The deletion or mutation of Glu^{263} abolishes TAP function, whereas the deletion or mutation of Phe^{265} influences peptide transport specificities rather than peptide binding, proposing functional differences between both amino acids. In contrast to previous studies, recent data show that the TAP1-mediated ATP hydrolysis is not essential for peptide translocation, but is involved in later stages of the translocation cycle or in controlling TAP activity (19). Furthermore, there exist functional differences between the nucleotide binding domains of the TAP1 and TAP2 subunits regarding the distinct steps of the TAP transport cycle (18, 19). The early events in the peptide transport produce conformational changes in TAP. This may be associated with a coordinated crosstalk among ATP binding, ATP hydrolysis, peptide binding,

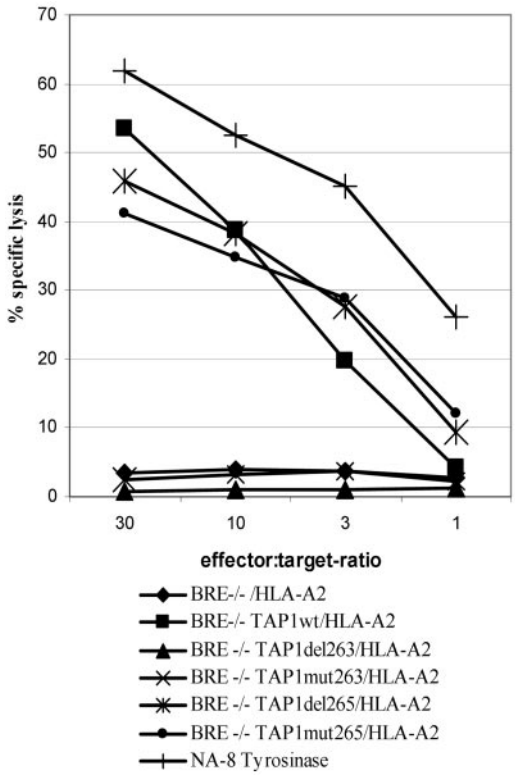


FIGURE 6. TAP1 del/mut 263 are not recognized by tyrosinase-specific CTL. Vaccinia tyrosinase-infected BRE TAP1 mut/del cells served as targets in a standard chromium release assay with tyrosinase-specific CTL using different E:T ratios. Tyrosinase-positive NA8 melanoma cells served as controls. All experiments were performed at least three times.

and translocation (16, 31). Further studies aimed at confirming this assumption by both peptide translocation and binding assays using a series of model peptides and by functional analyses of Glu^{263} and Phe^{265} replacements by different amino acids are currently performed. The distinct biological properties of Glu^{263} and Phe^{265} of the TAP1 subunit further support the hypothesis that structural features of members of the ABC transporter family provide information about amino acids or domains critical for TAP function. Thus, sequence similarities may lead at least in some cases to the prediction of motifs important for substrate specificity and transporter function.

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References

1. Monaco, J. J., S. Cho, and M. Attaya. 1990. Transport protein genes in the murine MHC: possible implications for antigen processing. *Science 250:1723.*
2. Pastan, I. H., and M. M. Gottesmann. 1988. Molecular biology of multidrug resistance in human cells. *Important Adv. Oncol. 5:3.*
3. Riordan, J. R., and X. B. Chang. 1992. CFTR, a channel with the structure of a transporter. *Biochim. Biophys. Acta 1101:221.*
4. Nunes, V., M. Chillon, T. Dörk, B. Tümmler, T. Casals, and X. Estivill. 1993. A new missense mutation (E92K) in the first transmembrane domain of the CFTR gene causes a benign cystic fibrosis phenotype. *Hum. Mol. Genet. 2:79.*
5. Schaedel, C., A. Kristoffersson, R. Kornfält, and L. Holmberg. 1994. A novel cystic fibrosis mutation, Y109C, in the first transmembrane domain of CFTR. *Hum. Mol. Genet. 3:1001.*
6. Sheppard, D. N., D. P. Rich, L. S. Ostedgaard, R. J. Gregory, A. E. Smith, and M. J. Welsh. 1993. Mutations in CFTR associated with mild-disease-form Cl^- channels with altered pore properties. *Nature 362:160.*
7. Loo, T. W., and D. M. Clarke. 1993. Functional consequences of phenylalanine mutations in the predicted transmembrane domain of P-glycoprotein. *J. Biol. Chem. 268:19965.*
8. Beaudet, L., I. L. Urbatsch, and P. Gros. 1998. Mutations in the nucleotide-binding sites of P-glycoprotein that affect substrate specificity modulate substrate induced ATP activity. *Biochemistry 37:9073.*
9. Neefjes, J. J., F. Momburg, and G. J. Hämmerling. 1993. Selectivity and ATP-dependent translocation of peptides by the MHC-encoded transporter. *Science 261:769.*
10. Russ, G., F. Esquivel, J. W. Yewdell, P. Cresswell, T. Spies, and J. R. Bennink. 1995. Assembly, intracellular location and nucleotide binding properties of the human peptide transporters TAP1 and TAP2 expressed by recombinant vaccinia virus. *J. Biol. Chem. 270:21312.*
11. Uebel, S., T. H. Meyer, W. Kraas, S. Kienle, G. Jung, K. H. Wiesmüller, and R. Tampé. 1995. Requirement for peptide binding to the human transporter associated with antigen processing revealed by peptide scans and complex peptide libraries. *J. Biol. Chem. 270:18512.*
12. Nijenhuis, M., and G. J. Hämmerling. 1996. Multiple regions of the transporter associated with antigen processing (TAP) contribute to its peptide binding site. *J. Immunol. 157:5467.*
13. Vos, J. C., P. Spee, F. Momburg, and J. Neefjes. 1999. Membrane topology and dimerization of the two subunits of the transporter associated with antigen processing reveal a three domain structure. *J. Immunol. 163:6679.*
14. Ritz, U., F. Momburg, H. P. Pircher, D. Strand, C. Huber, and B. Seliger. 2001. Identification of sequences in the human peptide transporter subunit TAP1 required for transporter associated with antigen processing (TAP) function. *Int. Immunol. 13:31.*
15. Knittler, M. R., P. Alberts, E. V. Deverson, and J. C. Howard. 1999. Nucleotide binding by TAP mediates association with peptide and release of assembled MHC class I molecules. *Curr. Biol. 9:999.*
16. Gorbulev, S., R. Abele, and R. Tampe. 2001. Allosteric crosstalk between peptide binding, transport and ATP-hydrolysis of the ABC transporter TAP. *Proc Natl. Acad. Sci. USA 98:3732.*
17. Lapinski, P. E., R. R. Neubig, and M. Rhagavan. 2001. Walker A lysine mutations of tap1 and tap2 interfere with peptide translocation but not peptide binding. *J. Biol. Chem. 276:7526.*
18. Arora, S., P. E. Lapinski, and M. Raghavan. 2001. Use of chimeric proteins to investigate the role of TAP structural domains in peptide binding and translocation. *Proc. Natl. Acad. Sci. USA 98:7241.*
19. Karttunen, J. T., P. J. Lehner, S. S. Gupta, E. W. Hewitt, and P. Cresswell. 2001. Distinct functions and cooperative interaction of the subunits of TAP. *Proc. Natl. Acad. Sci. USA 98:7431.*
20. Kallfelz, M., D. Jung, C. Hilmes, A. Knuth, E. Jaeger, C. Huber, and B. Seliger. 1999. Induction of immunogenicity of a human renal-cell carcinoma cell line by TAP1-gene transfer. *Int. J. Cancer 81:125.*
21. Van Kaer, L., P. G. Ashton-Rickardt, H. L. Ploegh, and S. Tonegawa. 1992. TAP1 mutant mice are deficient in antigen presentation, surface class I molecules, and $CD4^-8^+$ T cells. *Cell 71:1205.*
22. De la Salle, H., J. Zimmer, D. Fricker, C. Angenieux, J. P. Cazenave, M. Okubo, H. Meada, A. Plebani, M. M. Tongio, A. Dormoy, and D. Hanau. 1999. HLA class I deficiencies due to mutations in subunit 1 of the peptide transporter TAP1. *J. Clin. Invest. 103:R9.*
23. Wölfel, T., J. Schneider, K. H. Meyer zum Buschenfelde, H. G. Rammensee, O. Rotzschke, and K. Falk. 1994. Isolation of naturally processed peptides recognized by cytolytic T lymphocytes on human melanoma cells in association with HLA-A2.1. *Int. J. Cancer 57:413.*
24. Meyer, T., P. M. van Endert, S. Uebel, B. Ehring, and R. Tampé. 1994. Functional expression and purification of the ABC transporter complex associated with antigen processing (TAP) in insect cells. *FEBS Lett. 351:443.*
25. Neefjes, J., E. Gottfried, J. Roelse, M. Grommé, R. Obst, G. J. Hämmerling, and F. Momburg. 1995. Analysis of fine specificity of rat, mouse and human TAP peptide transporters. *Eur. J. Immunol. 25:1133.*
26. Bernhard, H., J. Karbach, T. Wolfel, P. Busch, S. Storkel, M. Stockle, C. Wolfel, B. Seliger, C. Huber, and K. H. Meyer zum Buschenfelde. 1994. Cellular immune response to human renal cell carcinomas: definition of a common antigen recognized by HLA-A2-restricted cytotoxic T-lymphocytes (CTL). *Int. J. Cancer 59:837.*
27. Drexler, I., E. Antunes, M. Schmitz, T. Wölfel, C. Huber, V. Erfle, P. Rieber, M. Theobald, and G. Sutter. 1999. Modified vaccinia virus Ankara for delivery of human tyrosinase as melanoma associated antigen: induction of tyrosinase- and melanoma-specific human leukocyte antigen AA*0201-restricted cytotoxic T-cells in vitro and in vivo. *Cancer Res. 59:4955.*
28. Taguchi, Y., K. Kino, M. Morishima, T. Komano, S. E. Kane, and K. Ueda. 1997. Alteration of substrate specificity by mutations at the His61 position in predicted transmembrane domain 1 of human MDR1/P-glycoprotein. *Biochemistry 36:8883.*
29. Loo, T. W., and D. M. Clarke. 1994. Mutations to amino acids located in predicted transmembrane segment 6 modulate the activity and substrate specificity of human P-glycoprotein. *Biochemistry 33:14049.*
30. Yewdell, J. W., F. Esquivel, D. Arnold, T. Spies, L. C. Eisenlohr, and J. R. Bennink. 1993. Presentation of numerous viral peptides to mouse MHC class I restricted T lymphocytes is mediated by the human MHC-encoded transporter or by a hybrid mouse-human transporter. *J. Exp. Med. 177:1785.*
31. Saveanu, L., S. Daniel, and P. M. van Endert. 2001. Distinct functions of the ATP binding cassettes of transporters associated with antigen processing: a mutational analysis of Walker A and B sequences. *J. Biol. Chem. 276:22107.*