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Functional Analysis by Site-Directed Mutagenesis of the Complex Polymorphism in Rat Transporter Associated with Antigen Processing

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The transporter associated with Ag processing, TAP, is an endoplasmic reticulum resident heterodimeric member of the ATP-binding cassette transporter family. TAP transports short peptides from cytosol to the endoplasmic reticulum lumen for loading into recently synthesized class I MHC molecules. In the rat, two alleles of the TAP2 chain differ in their permissiveness to the transport of peptides with small hydrophobic, polar, or charged amino acids at the C terminus, and this correlates with differences between the peptide sets loaded into certain class I molecules in vivo. We have used segmental exchanges and site-directed mutagenesis to identify the residues in rat TAP2 responsible for differential transport between the two alleles of peptides terminating above all in the positively charged residue, arginine. Of the 25 residues by which the two functional TAP2 alleles differ, we have localized differential transport of peptides with a C-terminal arginine to two adjacent clusters of exchanges in the membrane domain involving a total of five amino acids. Each cluster, transferred by site-directed mutagenesis from the permissive to the restrictive sequence, can independently confer on TAP a partial ability to transport peptides with arginine at the C terminus. The results suggest that the permissive TAP2-A allele evolved in at least two steps, each partially permissive for peptides with charged C termini. The Journal of Immunology, 1998, 160: 2767–2779.

TAP, the transporter associated with Ag processing, is an endoplasmic reticulum (ER) resident multispanning membrane protein consisting of a nonglycosylated heterodimer, both polyproteins of which are members of the ATP-binding cassette (ABC) transporter family and are encoded in the class II region of the MHC (1). The adaptive function of TAP appears to be to transfer peptides, generated by the proteolysis of cytosolic substrates, into the ER where they are available for loading into nascent class I MHC glycoproteins (2, 3). Thus, cells genetically deficient in TAP function cannot load peptides derived from cytosolic substrates into class I MHC molecules, resulting in greatly reduced surface expression of class I, and the presence of empty class I molecules in the ER (4–7). Humans genetically deficient in TAP1 suffer from a functional immunodeficiency that results in an increased susceptibility to infectious disease (8). Reduced class I expression associated with immune evasion in human tumors can be caused by a somatic mutation resulting in defective TAP function (9). In addition, assays in vitro using either permeabilized cells or microsomal vesicles have shown directly that TAP is able to transport short peptides from the cytosolic to the luminal side of the ER membrane, in the presence of hydrolyzable ATP (10, 11).

Despite considerable research effort, the mechanism of transport and control of specificity in the superfamily of ABC transporters remains enigmatic. TAP is the only well-described eukaryotic transporter of this family with a low m.w. water-soluble substrate that can be indefinitely varied in structure, and this property makes TAP a potentially valuable model with which to study the principles of ABC transporter function generally. Elegant and simple in vitro assays (10, 11) have made it possible to analyze the transport specificity of TAP in some detail. The permissiveness of TAP is greatest for peptides in the size range of 8 to 11 amino acids (10, 12–15), which corresponds well to the optimum length for peptides that are loaded into the peptide binding groove of class I molecules (16). Peptides shorter than seven residues are generally transported poorly, while peptides longer than about 13 amino acids in length are transported erratically, some well, some poorly, some not at all. However, the permissiveness of TAP for peptides in the ideal size range is subject to genetic variation detectable by in vitro assays, for example between humans and the mouse. The variation focuses on permissiveness with respect to the C-terminal residue of the peptide. Human TAP is generally permissive for transport of 9-mer peptides (17, 18), while mouse TAP excludes peptides with polar or charged residues at the C terminus (14, 17, 18). In the rat, a favorable situation has been described in which permissive and restrictive TAPs apparently corresponding closely in specificity to the human and mouse TAPs, respectively, occur as a dimorphism (17, 19, 20). Approximately half of known laboratory rat MHC haplotypes carry...
a permissive TAP, while the other half carry a restrictive TAP (21). Furthermore, the functional difference between the two morphs is invested exclusively in the TAP2 polypeptide (19, 22). In an earlier report, we demonstrated that the functional polymorphism in rat TAP2 was correlated with a complex nucleotide sequence polymorphism resulting in 25 amino acid exchanges in the 703 amino acid-long TAP2 polypeptide (Ref. 19, and see Fig. 1C). A further 23 noncoding nucleotide exchanges were also linked to the functional polymorphism. We additionally noted that the majority of the nucleotide exchanges were in the N-terminal two-thirds of the polypeptide, which contains a number of hydrophobic segments presumably participating in

FIGURE 1. A, Construction of TAP2* sequence and TAP2*/TAP2* shuffle variants. Organization of TAP2* clone 441-11 showing key restriction sites used in the construction of TAP2* and TAP2 a+u and TAP2 u+TAP2 a shuffle constructs, and primers used for the amplification of 5' and 3' segments of TAP2* by RT-PCR. Base numbering in A and B is according to the published sequence of TAP2* (EMBL accession no. X63854). A detailed account of the cloning strategy is given in Materials and Methods. B, Site-directed mutagenesis of TAP2*. Mutagenic oligonucleotides used in pALTER for the construction of mut1 to mut4 sequences based on TAP2* (GenBank accession no. X75307). In each case, the mutagenic oligonucleotide transferred a segment of TAP2 a sequence into a TAP2* framework. C, Diagrams of the TAP2 a and TAP2* polypeptides with polymorphic residues itemized in single letter code. The four residue clusters corresponding to mut1, mut2, mut3, and mut4 are indicated in brackets. The arrow between R353 and S374 of the TAP2 a sequence indicates the cleavage point corresponding to the Nar1 site defining the 5' and 3' fragments involved in the end-shuffle constructs. D, Diagrams of all the shuffle and mutant constructs described in this study. Segments corresponding to TAP2 a are filled in, segments corresponding to TAP2* are left open. Individual TAP2 a-derived mutant residues are itemized in single letter code. All site-directed mutant constructs were prepared with both TAP2* and TAP2 C-terminal segments, as indicated.
the formation of a transmembrane channel for peptide translocation.

A detailed study assaying transport of C-terminal variant peptides in permeabilized cells in vitro recently identified two polymorphic regions of the TAP2 chain as of significance in determining transport specificity (23). In the present study, we show results of a survey using segmental exchange and site-directed mutagenesis in a mixture of in vitro and in vivo assays to identify the polymorphic residues of TAP2 mainly involved in the efficient transport of peptides terminating in arginine. The significance of C-terminal arginine in this system is the strong preference shown by the rat class I MHC molecule, RT1.Aa, for this amino acid at the C terminus of efficiently loaded peptides, a preference supported in the RT1I haplotype by the cis-encoded permissive TAP2a polypeptide (24, 25). It is indeed likely that most of the class I alleles associated in cis with permissive TAPA alleles have a preference for loading peptides with a positively charged C-terminal residue (37). Our results confirm the earlier study in emphasizing the importance of the AE dipeptide at positions 217 and 218 for the permissive phenotype. However, our results differ from Momburg et al.’s earlier study (23) in that we find that a three-residue exchange at positions 262, 265, and 266 also contributes to differential transport of peptides with a C-terminal arginine, while the exchange at 374 and 380, which was found to be significant in the earlier study, is not apparent in our analysis. Thus, with respect to the transport of peptides carrying arginine at the C terminus, we have been able to locate most, if not all, of the activity of the two morphs of rat TAP2 to two adjacent clusters of amino acid exchanges in the putative membrane-spanning regions of the molecule. We additionally show that polymorphic residues associated with differential transport alter the specificity of TAP in vivo, and thereby affect the spectrum of peptides loaded into a classical class I MHC molecule.

Materials and Methods

Cell lines, tissue culture, and metabolic labeling

The normal growth medium for tissue culture was RPMI 1640 containing penicillin (50 U/ml), streptomycin (50 U/ml), l-glutamine, and 5% FCS.

The cell line C58.331 was made by cotransfection of the hypoxanthine-aaminopterin-thymidine (HAT)-sensitive rat thymoma, W/Fu(C58NTD), with a full-length cDNA encoding the rat classical class I molecule RT1.Aa from the DA strain expression plasmid pMSD and with a cDNA encoding hypoxanthine guanine phosphoribosyl transferase also in pMSD (26). This cell line and its derivatives by further transfection (see next paragraph) were maintained in HAT medium throughout the experiments. In the present text we refer to C58.331 as C58.RT1Aa for clarity.

For transfection of TAP2 sequences in the expression vector pHβApr-1-neo (see below) into C58.RT1Aa, aliquots of purified plasmid DNA were linearized at the unique vector Ndel site and transfected in 5- to 10-μg quantities by electroporation at 110 V, 120 V, and 140 V and 900 μF Farad using a Bio-Rad (Bio-Rad Laboratories Ltd., Hemel Hempstead, U.K.) cell electroporator. Transfectants were selected by growth of the electroporated cells in G418 at 1 μg/ml.

The maturation of RT1.Aa glycans during biosynthesis was analyzed by pulse-chase procedures essentially according to Powis, Howard, and Butcher (27). Cells were labeled at 100 μCi/ml in 2-ml vol (20 × 10^6/ml) with TRAN'S-LABEL (ICN Biomedicals, Thame, U.K.; sp. act., >1000Ci/mmol) for 15 min followed by cold chase. RTI.Aa was immunoprecipitated from Nonidet P-40 lysates of labeled cells with MAC30 (see next paragraph), and protein A-Sepharose. The immunoprecipitates were heated in SDS sample buffer containing 1.4 M 2-ME and run on 13% SDS-polyacrylamide gels. After soaking for 15 min in Amplify (Amerham International plc, Amersham, U.K.) the gels were dried down and exposed on Fuji X100 film at -70°C.

mAbS AND FACS analysis

The following mAbs, described in Reference 28, were employed in the experiments described in this study: R3/13 (rat IgG2b), MAC30 (rat IgG2c), and JY3/84 (rat IgG2a) directed against distinct epitopes of the rat classical class I histocompatibility Ag RT1.Aa, and NR5/10 (rat IgG2b) directed against the rat classical class I histocompatibility Ag RT1.Aa.

For analysis of expression of rat class I MHC molecules on the surface of C58.RT1Aa cells and their transfectant derivatives, cells were harvested from tissue culture, washed in cold PBS containing FCS (2% v/v) and sodium azide (0.1% v/v), and incubated with shaking at 4°C with tissue culture supernatants of the rat thymoma lines R3/13, JY3/84, and R3/14 binding, bound Ab was detected on live cells after a further incubation with fluorescein-labeled rabbit anti-rat IgG (DAKO Ltd., High Wycombe, U.K.) in a FACSScan (Becton Dickinson Immunocytometry Systems Europe, Embegem, Belgium) using propidium iodide as a counterstain to identify and exclude dead cells. In general, serologic assays were used for preliminary screening of transfectant populations and clones because of the tell-tale shift in JY3/84 expression associated with TAP2a function (29, 30).

Generation and assay of cytotoxic T cells directed against RT1.Aa+

RT1.Aa is expressed on the surface of rat cells in distinct antigenic forms, designated A+a and A+a, depending on which allele of TAP2 is present (29, 30). Immunization between strains of rat carrying different forms of RT1.Aa generates CTL populations specific for the two antigenic forms. Detailed procedures for the generation and assay of CTL specific for RT1.Aa were essentially as previously described (27, 30). To raise CTL specific for RT1.Aa+, PVG.R1 (RT1.Aa+) rats were immunized with PVG.R1 (RT1.Aa+) splenocytes. Lymph node cells were subsequently restimulated in vitro with irradiated PVG.R1 spleno
cytes. Assay of CTL activity was on 35S-labeled PVG.R19 or appropriate control Con A blast targets. Expression of RT1.Aa+ epitopes on transfectants of C58.RT1Aa was assayed by cold-target competition against labeled PVG.R19 Con A blasts. Cold-target competitor cells were added at ratios of 100:1, 33:1, and 11:1 relative to hot targets.

TAP2 shuffle exchanges and site-directed mutagenesis

TAP2a and TAP2b. For genetic manipulations, TAP2b was obtained as an EcoRI fragment from the plasmid pBS441-11 that contains a TAP2 cDNA derived from a DA strain lymphoblast (R2) line. The full-length TAP2b cDNA was obtained as follows: overlapping 5′ and 3′ fragments of TAP2b were prepared by reverse transcriptase (RT)-PCR from poly(A) RNA derived from Con A blasts of PVG.R8 origin using the strategy and primers shown in Figure 1A. Independent fragments were cloned into pBluescript KS(+) and sequenced (6, 21). The 5′ NcoI-NarI fragment and 3′ NarI-SnaI fragments were ligated in a single step into pBluescript containing the TAP2a cDNA previously cut with NcoI and SnaI to excise the whole coding sequence. The full-length TAP2b construction including 5′ and 3′ untranslated regions from TAP2b was then excised with EcoRI and transferred into the correct orientation into the expression vector pHβApr-1-neo. This construct, known as clone no. 4, which has not been described in detail previously, was used to reconstitute the human TAP-deficient cell line, thereby documenting its functionality (17).

TAP2a-u and TAP2a-u shuffled constructs. Two constructs were prepared in which the TAP2a and TAP2b sequences were shuffled at the NarI site (Fig. 1A, bp 1191). To prepare TAP2a-u, the 5′ Ncol-NarI fragment of TAP2a was cloned into Ncol/NarI-digested full-length TAP2b in pBluescript prepared as above. NarI cleaves near the middle of the TAP2 sequence, leaving 17 polymorphic amino acid residues coded by the 5′ sequence and 8 polymorphic amino acid residues coded by the 3′ sequence (Fig. 1C). Using the original EcoRI cloning sites, the full-length recombinant clones were transferred from pBluescript to pHβApr-1-neo for transfection.

Site-directed mutagenesis. The clone containing the 5′ half of TAP2b used for the full reconstruction described above (derived from RT-PCR with primers A and B; see Fig. 1A) was digested with EcoRI and XhoI using the restriction sites engineered into the primers and transferred to pALTTER (Promega Corporation, Madison, WI). Mutagenesis using specific mutagenic primers proceeded according to the manufacturer’s instructions. After confirmation of the sequence of the mutagenized region, the Ncol/NarI 5′ fragment was transferred to an appropriate “host” 3′ TAP2a or TAP2b construct in pBluescript as described above for the formation of the shuffle constructs. Figure 1B shows the four mutagenic primers used to construct the mutant TAP2 molecules described in this study. In each case, complete “motifs” of allele-specific sequence (Fig. 1C) were replaced, resulting in the exchange of two or three amino acids. The exchange of multiple motifs generating mut(1-2) and mut(1+2+3) was achieved by performing the mutagenic strand reconstruction using several mutagenic primers simultaneously. The full set of mutagenized constructs used in this study was constructed using the mutagenic strand reconstruction described in Methods.
study is depicted in Figure 1D. After construction in pbLUscript, all muta-
ted full-length TAP2 sequences were transferred to pHβAPr-1-neo for transfection.

Isolation of peptides and HPLC
For metabolic labeling preceding isolation of labeled peptides from immu-
noprecipitated class I molecules, cells were concentrated to 5 × 10^6/ml in 10 ml of leucine-free RPMI 1640 (Selectamine Kit; Life Technologies Ltd., Basingstoke, U.K.) containing HAT, G418, and 0.5 μCi of [3H]leucine (TRK683; Amersham International plc), and incubated for a further 10 to 12 h. During this period, >95% of the free label was depleted from the supernatant. Aliquots of 50 × 10^6 [3H]leucine-labeled cells were handled essentially according to Van Bleek and Nathenson (31) to isolate peptides dried at 37°C overnight before the addition of scintillant. Samples were added to the cells and incubated for 15 min at 37°C. Cells were lysed in 1 ml of Con A binding buffer (500 mM NaCl, 20 mM HEPES, pH 7.5, 1 mM Pefabloc (Boehringer Mannheim U.K. Ltd., Lewes, U.K.), 1 mM PMSF, pH 7.5 before affinity chromatography on an immobilized anhydrotrypsin col-
umn (Fischer, Rust, Germany). Washed, immunoabsorbent-bound material was eluted by boiling in 10% acetic acid and the supernatant transferred to a Centricon 10 (Ami-
con Inc., Beverly, MA) ultrafiltration unit. The filtrate was subjected to reverse-phase chromatography using a SMART HPLC system (Pharmacia Biotech, Uppsala, Sweden). Washed, immunoabsorbent-bound material was eluted by boiling in 10% acetic acid and the supernatant transferred to a Centricon 10 (Ami-
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con Inc., Beverly, MA) ultrafiltration unit. The filtrate was subjected to reverse-phase chromatography using a SMART HPLC system (Pharmacia Biotech, Uppsala, Sweden).

Peptides eluted in 10% acetic acid as described above were adjusted to pH 5.0 before affinity chromatography on an immobilized anhydrotrypsin col-
umn (Pierce, Rockford, IL). The column was washed with 6 ml of binding buffer (0.05 M sodium acetate, 0.02 M calcium chloride, 0.05% (w/v) sodium azide, pH 5.0) to remove unbound peptides, and bound peptides were then eluted using 6 ml of elution buffer (0.1 M formic acid, pH 2.5).

In vitro peptide transport assay
The nonamer peptides TVDNKTRYR and TVDNKTRYV (17) were syn-
mthesized using FMOC-Arg or BOC-Val resin by the Microchemical Facil-
ity, The Netherlands, U.K.) and assembled on the SMART column. The SMART column was equilibrated with 0.1 M formic acid, pH 2.5. The bound peptides were then eluted using 6 ml of elution buffer (0.1 M formic acid, pH 2.5). Both bound and unbound peptide fractions were dried and redissolved in 10% acetic acid before chromatography on an Aquapore 300 HPLC col-
umn as described above.

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umn as described above.

Anhydrotrypsin affinity columns
Peptides eluted in 10% acetic acid as described above were adjusted to pH 5.0 before affinity chromatography on an immobilized anhydrotrypsin col-
umn (Pierce, Rockford, IL). The column was washed with 6 ml of binding buffer (0.05 M sodium acetate, 0.02 M calcium chloride, 0.05% (w/v) sodium azide, pH 5.0) to remove unbound peptides, and bound peptides were then eluted using 6 ml of elution buffer (0.1 M formic acid, pH 2.5).

Both bound and unbound peptide fractions were dried and redissolved in 10% acetic acid before chromatography on an Aquapore 300 HPLC col-
umn as described above.

Results
Amino-carboxyl-terminal exchange between TAP2α and TAP2β
The differential amino acids distinguishing the permissive TAP2α and restrictive TAP2α polypeptides are indicated in Figure 1C. All 25 allele-specific amino acid exchanges were initially candidates for the documented functional differences between the two TAP2 groups. To simplify the problem, we therefore generated amine-
carboxyl-terminal exchanges, Tap2α+u and Tap2 u u, between TAP2α and TAP2β, based on a shared Nar1 restriction site that cleaves the cDNA immediately C-terminal to a tryptophan residue at position 367 in the translated amino acid sequence. Of the 25 function-correlated amino acid exchanges, 17 are included in the N-terminal segment generated by this digest, and 8 in the C-terminal segment. The swapped TAP2 chains were cloned into an expression vector and stably transfected into the C58.RT1.Aα cells (29, 30). The expression of RT1α + in the C58.RT1.Aα cell line was assayed by FACS using two TAP2-specific mAbs, R3/13 and JY3/84. R3/13 detects all TAP2α alleles, while JY3/84 detects an epitope on RT1α + preferentially expressed when a permissive transporter is present (29, 30). Individual clones of cells were subsequently isolated from these and other primary transfectant populations. The positive and negative control clones, C58.RT1.Aα.B5 (TAP2α) (19) and C58.RT1.Aα.D7 (TAP2β) were used in subsequent experiments as controls for permissive and restrictive TAP2 expression, respectively.

Enhanced expression of R3/13 and JY3/84 indicating TAP2α-like activity was observed in uncloned lines (Fig. 2A) and cell clones (Fig. 2B) carrying the TAP2α and α-u constructs but not in

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those carrying the TAP2\(^a\) or u+a constructs, implicating the N-terminal substitutions in the differential function of TAP2\(^a\). This preliminary result was confirmed on individual cloned lines by all the analytical criteria by which TAP2\(^a\)-loaded RT1.A\(^a\) can be distinguished from the TAP2\(^u\)-loaded form.

Figure 3 shows profiles of internally labeled peptides eluted from RT1.A\(^a\) molecules immunoprecipitated from C58.RT1.A\(^a\) transfectants carrying a, u, a+u, and u+a TAP2 chains. The bulk of early eluted material between fractions 20 and 60 (approximately) characteristic of cells expressing TAP2\(^u\) and a+u chains contrasts with the flat profiles in this region interrupted by two abrupt peaks around fractions 32 and 60, characteristic of cells expressing TAP2\(^a\) and u+a chains. However, no distinction could be made between "a" and "a+u" elution profiles on the one hand, or between "u" and "u+a" elution profiles on the other. Inconsistent profiles beyond fraction 70 are typical of elutions of RT1.A\(^a\)-associated peptides from C58 transfectants; we have to date detected no correlation between features of the peptide profiles beyond fraction 70 and the genetic identity of the TAP2 chain.

The characteristically different peptide sets loaded into RT1.A\(^a\) in the presence of TAP2 chains with a TAP2\(^a\)- or TAP2\(^u\)-derived sequence at the N-terminal segment were also reflected in the expression on transfectant cells of the distinct target determinants for CTL lysis through RT1.A\(^a\) associated with TAP2\(^a\) and TAP2\(^u\). C58.RT1.A\(^a\) transfectants carrying wild-type, recombinant, and mutant TAP2 chains were used as cold targets in an inhibition assay (see Materials and Methods) based on the lysis of 51Cr-labeled Con A blasts derived from PVG.R19 rats expressing the RT1.A\(^a+\) CTL specificities associated with the permissive TAP2\(^a\) transporter. Thus, the expression of full or partial sets of RT1.A\(^a+\) specificities by the different transfectants could be detected. Figure 4 shows that in cold-target competition, cells carrying TAP2a+u chains (panel B) faithfully reproduce the strong competitive activity for RT1.A\(^a+\) of transfectants carrying TAP2a (panel A), while cells carrying TAP2\(^u\) (panel A) and u+a (panel B) chains have no detectable competitive activity for RT1.A\(^a+\) (see also panels C-E).

As in previous reports (19, 27, 29), the expression of TAP2\(^a\) was associated with rapid transit of newly synthesized RT1.A\(^a\) molecules through the Golgi, as witnessed by rapid glycan maturation during pulse chase, detected by decreased mobility in SDS-PAGE (Fig. 5). Similarly rapid maturation of RT1.A\(^a\) was also seen in cells transfected with the TAP2a+u construct, while typical slow RT1.A\(^a\) maturation was seen in cells transfected with TAP2\(^u\) or u+a constructs.

Finally, in a direct transport assay (Fig. 6A), a test 9-mer peptide carrying arginine at the C terminus was efficiently transported into the ER in permeabilized cells carrying the TAP2\(^a\) and a+u constructs, while the peptide was effectively not transported by cells carrying the TAP2\(^u\) and u+a constructs.

**Site-directed exchange mutagenesis of allele-specific sequence motifs in the N-terminal segment of TAP2**

To localize the residues responsible for differential function more precisely, we performed exchange mutations by site-directed mutagenesis in the N-terminal segment of the TAP2 chain (Fig. 1, B-D). We focused on the four well-marked clusters of allelic substitutions located in the N-terminal segment of the TAP2 sequence whose position and character had already attracted our attention (19). Because C58.RT1.A\(^a\) already carries an endogenous TAP2\(^a\) allelic sequence, motifs were transferred from TAP2\(^a\) to TAP2\(^u\), thus searching for a positive function in a negative background. To explore whether the C-terminal segment of the molecule, apparently inactive according to the results of the end-shuffles (see above), was able to contribute

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**FIGURE 2.** A. FACS plots of immunofluorescence profiles assaying RT1.A\(^a\) expressed on uncloned populations of C58.RT1.A\(^a\) cells transfected with (from top to bottom) TAP2\(^a\), TAPu+a, TAP2\(^u\), and TAP2a+u constructs. The three profiles for each population correspond to (left to right) PBS containing FCS (2% v/v) and sodium azide (0.1% w/v) (negative control), JY3/84 (bold line), and R3/13 (positive control). Each profile contains 10,000 cells, plotted as cell number per channel (ordinate) against immunofluorescence units on a logarithmic scale. B. Correlated mean JY3/84 and R3/13 fluorescence of individual clones of C58.RT1.A\(^a\) transfectants isolated from the uncloned populations analyzed in Figure 1A. Higher expression of RT1.A\(^a\) (measured by R3/13 fluorescence) and of the RT1.A\(^a+\)-associated determinant JY3/84 is apparent in transfectants containing TAP2\(^a\) and the TAP2a+u shuffle construct. Clones containing the TAP2 u+a shuffle construct are indistinguishable from those carrying TAP2\(^a\).
FIGURE 3. Elution profiles of counts recovered from reversed-phase HPLC of \[ ^{3}H \]leucine-labeled peptides isolated from RT1.A\(^{a} \) molecules derived from cells carrying different versions of TAP2. A, RT1.A\(^{a} \) molecules were immunoprecipitated from transfectant C58.RT1.A\(^{a} \) cell populations carrying constructs expressing wild-type or end-shuffled TAP2 chains as indicated. Two independent profiles are shown to represent each construct. B, RT1.A\(^{a} \) molecules were immunoprecipitated from transfectant C58.RT1.A\(^{a} \) cell populations carrying constructs expressing wild-type TAP2\(^{a} \) or TAP2\(^{u} \) chains, or TAP2\(^{u} \) chains with site-directed mutations giving TAP2\(^{a} \) sequence at the mut1, mut(1+2), or mut(1+2+3) polymorphic site clusters as indicated (see also Fig. 1D). The identity of the C-terminal TAP2 segment in each construct is also indicated; thus u(1+2)+u identifies mut1 and mut2 from TAP2\(^{u} \) in the N-terminal segment of TAP2\(^{a} \) associated with the C-terminal segment of TAP2\(^{u} \). Two independent profiles are shown to represent each of the mutant constructs. C, RT1.A\(^{a} \) molecules were immunoprecipitated from transfectant C58.RT1.A\(^{a} \) cell populations carrying wild-type TAP2\(^{a} \) or TAP2\(^{u} \) chains, or TAP2\(^{u} \) chains with site-directed mutations giving TAP2\(^{a} \) sequence at the mut4 polymorphic site cluster (see also Fig. 1D). The identity of the C-terminal TAP2 segment in each construct is indicated as in B.
cooperatively to the specificity of peptide transport, all mutant N-terminal TAP2″ segments were cloned with both TAP2 a and TAP2 u C-terminal segments (see Fig. 1D).

Mutation 1 (mut1), consisting of the replacement only of the two residues TM(217-218) in TAP2 u with AE(217-218) from TAP2 a, had a large effect on the transport properties of the TAP2 u chain, an effect close in magnitude, if not quite equal, to the whole of the N-terminal segment from TAP2 a. This could be seen as elevated expression levels of the R3/13 and JY3/84 epitopes of RT1.A a (data not shown), rapid maturation of glycans (Fig. 5), the presence

![Figure 4](https://example.com/fig4.png)

**FIGURE 4.** Expression of the TAP2 a-dependent RT1.A a form of RT1.A a on the surface of TAP2-transfected C58.RT1.A a clones, detected by cold-target inhibition of cell-mediated cytotoxicity using RT1.A a-specific CTL and 51Cr-labeled PVG.R19 (RT1.A a) Con A blast targets as described in Materials and Methods. Lines grouped in square brackets depict data from sister clones transfected for the same TAP2 construct. Each experiment was repeated three times with CTL populations derived from different rats. A and B, Cold targets were C58.RT1.A a clones transfected with wild-type TAP2 a and TAP2 u constructs (A) and with TAP2 u+a and TAP2 a+u end-shuffle constructs (B). The results on the two panels were from a single experiment and can be directly compared. C, Cold targets were C58.RT1.A a clones transfected with mut1+a and mut1+u constructs of TAP2, compared with wild-type TAP2 a and TAP2 u transfectants. D and E, Cold targets were C58.RT1.A a clones transfected with mut1 and mut4 constructs of TAP2. mut1+u and mut1+u constructs. mut1+a and mut4+a constructs. Cold targets transfected with TAP2 a, TAP2 u, TAP2 a+u, and TAP2 u+a constructs were included as controls. The results on the two panels were from a single experiment and can be directly compared.

![Figure 5](https://example.com/fig5.png)

**FIGURE 5.** Maturation of RT1.A a heavy chain in individual clones of C58.RT1.A a cells transfected with wild-type, end-shuffle, or mut1 TAP2 chains assayed by biosynthetic labeling with pulse-chase. RT1.A a was immunoprecipitated from cells metabolically labeled in vitro with [35S]methionine/[35S]cysteine, followed by 0, 30, or 90 min of chase with unlabeled methionine/cysteine. The mature higher apparent m.w. form of RT1.A a is clearly visible at 30 min and is the only significant signal at 90 min of cold chase (arrowed) in cells transfected with wild-type TAP2 a, TAP2 a+u, and TAP2 mut1 constructs, while immature RT1.A a still gives a substantial signal even after 90 min of cold chase (arrowed) in cells transfected with wild-type TAP2 u or TAP2 u+a constructs.
in immunoprecipitates of RT1.A of a substantial yield of peptides eluting early from reverse-phase HPLC (Fig. 3B, panels iii, iv, v, vi), and levels of ATP-dependent transport of the C-terminal arginine test peptide into the ER comparable to the parental TAP2a or a/u shuffle construct (Fig. 6A). Unlike the TAP2a or a/u shuffle constructs, however, expression of the RT1.A+ CTL-defined TAP2a-dependent epitopes as measured by cold-target competition was relatively weak (Fig. 4, C-E), suggesting that a certain subset of peptides contributing significantly to the antigenic epitopes of RT1.A+ was poorly represented in the set efficiently transported by the mut1 TAP2 construct. The phenotype of the mut1 construct was not altered by adding mutant motifs 2 and 3 to mut1 (see Figs. 1 and 3B, panels vii-xii). In no case were any of the properties of the TAP2a allele reconstituted by exchange of the allelic motifs in mutations 2 or 3 alone, in association with TAP2u or TAP2a C-terminal segments of the transporter (data not shown). Likewise, the behavior of the mut1 construct was not significantly modified by replacement of the C-terminal segment of TAP2u with the corresponding segment of TAP2a in any assay (Figs. 3–6).

The mut4 construct, replacing RPF(262, 265, 266) from TAP2u with QSL(262, 265, 266) from TAP2a, in association with either TAP2u or TAP2a C-terminal segments, also showed a partial reconstitution of the TAP2a phenotype, which was however different from, and to a degree complementary to, that of the parent TAP2a allele, as measured by cold-target competition (Fig. 4, C-E).
shown by the mut1 constructs. In this case, the level of reconstitution of the TAP2*-dependent phenotype was relatively weak in terms of the modification of the total peptide set loaded into RT1.A* (Fig. 3C, panels iii, iv, v, and vi). A small (see below, Fig. 7) excess of peptide mass was consistently seen in the mut4 profiles relative to TAP2* transfectants starting in fractions earlier than the characteristic sharp peak at around fraction 33 and continuing in the region between the 33 peak and the fraction 60 peak. This quantitatively weak activity in loading the bulk population of peptides with arginine C termini into RT1.A* was also reflected in weak (Fig. 6A) though clearly significant (Fig. 6B) transport of the C-terminal arginine test peptide into the ER, and no evidence for rapid mobilization of RT1.A* out of the ER in terms of glycan modification (data not shown). Despite this quantitatively marginal activity, cells carrying mut4 constructs showed expression of RT1.A* epitopes defined by cold-target competition of RT1.A*-specific CTL that was generally higher than that shown by mut1 constructs (Fig. 4, D and E). These results carry the implication that mut4 constructs may be selectively permissive for a set of peptides that contribute disproportionately to the RT1.A* antigenic phenotype.

**FIGURE 7.** Binding of RT1.A*-derived peptides to anhydrotrypsin affinity columns. Elution profiles of counts recovered from reversed-phase HPLC of [3H]leucine-labeled peptides isolated from RT1.A* molecules. Panels show profiles for total recovered peptides (open profiles), peptides passed straight through an anhydrotrypsin affinity column (shaded profiles), and peptides eluted from the column (filled profiles), for individual C58.RT1.A* clones carrying wild-type TAP2* (i, iv), TAP2* (vii, x), mut1 constructs (ii, iii, v, vi) and mut4 constructs (viii, vii, xi xii) as indicated.
Anhydrotrypsin columns provide a convenient way of assessing the proportion of peptides carrying an arginine residue at the C-terminus that are loaded into class I MHC molecules. Most of the peptides loaded into RT1.A\(^a\) in cells carrying TAP2\(^a\) are retained by anhydrotrypsin columns and can be eluted with 0.1 M formic acid, pH 2.5, showing the presence of the C-terminal arginine that is characteristic of the peptide-binding motif of RT1.A\(^a\), while essentially no peptides recovered from RT1.A\(^a\) of cells carrying only the TAP2\(^a\) allele will bind anhydrotrypsin (25). We used this criterion to assess the extent to which the apparent reconstitution of transport of peptides carrying charged C-terminal residues by the recombinant and mutant TAP2 chains shown above was reflected in the loading into RT1.A\(^a\) of typical complex populations of peptides carrying C-terminal arginine. As shown in Figure 7, panels iii, iv, v, and vi, clones carrying the mut1 constructs load populations of anhydrotrypsin-binding peptides into RT1.A\(^a\) that are comparable in yield and complexity with those obtained from cells carrying the wild-type TAP2\(^a\) construct (Fig. 7, panels i and ii). As in the assays described in the previous section, there was no visible distinction between the profiles of anhydrotrypsin-binding peptides from cells with mut1 constructs expressing the TAP2\(^a\) (Fig. 7, panels iii and iv) or TAP2\(^a\) (Fig. 7, panels v and vi) C-terminal segments.

The profiles of anhydrotrypsin-bound and unbound peptides derived from mut4 transfectants (Fig. 7, panels ix, x, xi, xii) were much more similar to those of transfectants carrying only the wild-type TAP2\(^a\) allele (profiles vii and viii), as indeed were the total profiles, as shown in the previous section (Fig. 3c). However, in all cases, profiles from the mut4 transfectants showed a small excess of early eluting anhydrotrypsin-bound peptide material that presumably coincided with the small peptide excess noted from the mut4 total profiles shown above. This characteristic anhydrotrypsin profile was independent of the allelic identity of the C-terminal segment of TAP2 (compare Fig. 7, panels ix and x with Fig. 7, panels xi and xii) and consistent with the weakly permissive transport seen in the direct transport assay (Fig. 6, A and B).

Discussion

The two functionally distinct alleles of rat TAP2 have provided us with an opportunity to investigate the amino acid residues involved in differential peptide transport. TAP2 alleles of the TAP2-A (including TAP2\(^a\) used in this study) and TAP2-B groups (including TAP2\(^b\) used in this study) differ by 25 amino acid residues (19, 21). We have used segmental shuffling and site-directed mutagenesis to identify five of these, distributed in two short adjacent clusters, which appear to be of key significance in the differential transport of peptides carrying a C-terminal arginine. While the differential function of permissive alleles of the TAP2-A group and restrictive alleles of the TAP2-B group extends to small and polar amino acids as well as other charged amino acids at the peptide C-terminus, arginine appears to be of peculiar significance for the dimorphic function. The reason for this is that in the rat, the permissive allele appears to have been evolved to allow the efficient loading of class I MHC allelic products with unusual concentrations of negative charge in the peptide C-terminal binding pocket of the peptide binding groove (24). In the one case that has been well studied, namely the binding of peptides by the RT1.A\(^a\) allelic product, the peptide C-terminal binding pocket shows an overwhelming preference for arginine (25). In this case, the preference is so strong that when only the restrictive form of TAP is present, the resulting failure to deliver arginine-ended peptides to the ER lumen results in highly significant delays in RT1.A\(^a\) maturation and export to the cell surface (27). Our assays for the function of polymorphic residues in TAP2 focus on the transport of arginine-terminated peptides in three ways. Firstly, as stated, assays involving aspects of the expression or peptide loading of RT1.A\(^a\) in cells are largely limited to arginine-terminated peptides because of the loading bias of this class I molecule; secondly, in the direct transport assay we exclusively monitored the transport of a peptide terminating in arginine; and thirdly, we have used the arginine specificity of anhydrotrypsin to define the loaded peptide sets.

We have been able to show that two clusters of polymorphic residues are responsible for differential transport of R-terminated peptides. These are adjacent in the TAP2 chain at positions 217 and 218, and 262, 265, and 266. When these clusters are located on a hydrophobicity plot of TAP2 (Fig. 8a), they mark the two ends of a hydrophilic loop between two distinctly hydrophobic regions. This loop was named L2 and highlighted as a region of conserved structure between several ABC transporters in an earlier analysis (32). In the absence of direct experiment, there are good indirect reasons for placing the TAP2 217 to 266 L2 loop in the cytosol. These are based on sequence homology, the common hydrophobic structure of TAP chains, multidrug resistance polypeptide, MDR1, other related ABC transporters, and the placing of N-glycosylation sites and signals. The general conformity between hydrophobicity plots of MDR1 (N-terminal segment), TAP1, and TAP2 is apparent when the three polypeptides are aligned on the GKS Walker A motif in the nucleotide binding domain (Fig. 8a). This alignment is further validated by the direct sequence similarity in the L2 loop itself between MDR and TAP (Fig. 8b) exactly between TAP2 residues 217 and 266. It is almost certain that this loop of MDR normally sits in the cytosol. This is further supported by the presence of N-glycans in the N-terminal adjacent hydrophilic segment located on the other side of a hydrophobic presumed membrane-spanning region. The hydrophobicity plot of rat TAP1 is closely similar to that of TAP2, and the homologous hydrophilic segment is easy to locate. The only argument against this alignment and topology is the N-glycosylation signal in TAP1 at residues 227 to 229 underlined in Figure 8b: we have found no evidence for glycosylation at this site (A. Seelig, unpublished observations), consistent with a cytosolic location, but it is conserved in the homologous location in human and mouse TAP1. The particular interest in the homology of the specificity loop of TAP2 to the L2 loop of MDR1 is that the latter has been strongly implicated in drug transport by mutational studies (33). Two point mutations that affect the specificity of drug transport by MDR1 are in this loop (34, 35), and a further cluster of four MDR1 residues in the same loop is able to reconstitute MDR1 function when an 89-residue segment containing the corresponding loop from the non-drug-transporting human MDR2 gene is grafted into MDR1 (33, 35). It will be of great interest to determine whether the L2 loop of TAP2 is directly involved in interactions with peptide during TAP-mediated transport. A recent study using peptides carrying photoactivatable cross-linkers failed to identify positively the region of the L2 loop of TAP2 as significant in peptide binding (36). However, in this study the antipeptide antiserum raised against an epitope within the L2 loop was too low in titer to be of value. Direct information about the peptide binding activity of this segment of the TAP2 polypeptide could therefore not be obtained. Weak peptide cross-linking activity associated with a fragment identified by an antiserum binding N-terminal of the L2 loop was not shown to include the L2 loop itself. In view of the fact that both the mut1 and mut4 residues contribute to permissiveness of TAP2 for peptides with a C-terminal arginine, these two clusters may be adjacent in the folded structure of TAP. Their positions at the
extreme ends of the L2 loop, immediately adjacent to strongly hydrophobic segments, appears to place both the mut1 and mut4 amino acid clusters immediately adjacent to the membrane. The two polymorphic residue clusters appear to participate in transport of R-terminated peptides in qualitatively distinct ways. Thus the mut1 construct gives generally permissive transport for such peptides but fails,
at least in relative terms, to reconstruct the antigenic epitopes of the RT1.A<sup>+</sup> phenotype, while the weakly permissive mutant construct appears to reconstruct this antigenic phenotype with disproportionate efficiency (Fig. 4, B and C). It is therefore likely that the two polymorphic residue clusters at the ends of the L2 loop confer permissiveness to distinct subfamilies of R-terminated peptides. The nature of this fine structure within the frame of permissive transport is clearly of interest.

No functionally significant polymorphism has been detected in TAP from either humans or the mouse. However, the functional phenotype of human TAP corresponds to the permissive rat TAP2-A alleles while that of mouse TAP corresponds to the restrictive phenotype of rat TAP2-B alleles. As we pointed out earlier, the mouse TAP2 sequence in the 217 to 266 specificity loop corresponds to TAP2-B (Fig. 8C), consistent with the restrictive phenotype that these two transporters share. Surprisingly, however, permissive human TAP2 also has the restrictive TM sequence at 217 to 218 and shares only 266L with the permissive rat TAP2-A transporters. This result implies that the structural basis for permissive peptide transport by human and rat TAP2-A transporters, at least with respect to this key loop, must be different, providing an opportunity for further structure-function analysis.

The result also suggests, equally surprisingly, that permissive behavior of peptide transporters must have evolved twice within the three species studied. The time-depth of the sequence disparity between the rat TAP2-A and TAP2-B allelic groups is approximately half that between mouse and rat (L. Guethlein, unpublished observations), suggesting that the evolution of the divergent rat TAP2 alleles has been relatively recent, presumably reflecting co-adaptive evolution of a permissive TAP2 with rat class I alleles having a strongly acidic peptide C-terminal binding pocket.

Recently, another study of the residues involved in differential peptide transport by the rat TAP2 alleles appeared (23) in which a number of recombinant rat TAP2 chains with different clusters of polymorphic residues were analyzed for transport specificity in the direct assay also employed here (Fig. 6) but using peptides varying over the full range of C-terminal amino acids. As in our study, the mut1 dipeptide 217 to 218 (TM/AE) was highlighted as a determinant of differential transport, but significant differential transport activity was also localized to the polymorphic residues 374 and 380. In addition, a point mutation of human TAP2 residue 374 from alanine to aspartic acid resulted in some loss of permissive-ness again, suggesting a significant role for this region (22). Interestingly, the restrictive rat transporter also carries a negative charge at 374. Residues 374 and 380 are immediately C-terminal to the restriction enzyme cleavage site by which we shuffled N- and C-terminal segments of TAP2 (see Fig. 1), and we should therefore have expected that significant differential transport activity related to the 374 to 380 dimorphisms would have been detected not only in our shuffled construct, TAP2 u +/u, in which 374 to 380 are in the permissive configuration, but also in the mut4 constructs, where the weak transport activity associated with the mut4 cluster should have been significantly enhanced in mut4+a relative to mut4+u. In fact, by all our criteria the mut4+a and mut4+u constructs had indistinguishable activity. Part of the explanation for this apparent inconsistency may lie in the observation from Momburg and colleagues (23) that the 374 to 380 cluster is less relevant to permissiveness for C-terminal arginine than, for instance, glutamine or glutamic acid, while the mut1 cluster is strongly permissive for C-terminal arginine. It is nevertheless surprising that we failed to detect any arginine-permissive activity C-terminal of residue 267, not only by direct transport but also in assays of RT1.A<sup>+</sup>-loaded peptides in which detection is not limited to a single peptide species. It is equally surprising that Momburg et al. (23) failed to detect the mut4 cluster, which in our hands was unambiguously active in direct transport activity with the identical peptide (Fig. 6) as well as by all in vivo criteria (Figs. 3C, 4, and 7). Nevertheless, the two data sets together reiterate what we stated above, namely that the complex sequence divergence of the rat permissive TAP2 allele away from the restrictive allele appears to reflect the sum of a series of evolutionary steps, each responsible for distinctive aspects of the total permissive phenotype.

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**References**


