Brief Definitive Report

Presentation of Numerous Viral Peptides to Mouse Major Histocompatibility Complex (MHC) Class I-restricted T Lymphocytes Is Mediated by the Human MHC-encoded Transporter or by a Hybrid Mouse-Human Transporter

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Summary

The major histocompatibility complex-encoded transporter associated with antigen processing (TAP) is required for the efficient presentation of cytosolic antigens to class I-restricted T cells. TAP is thought to be formed by the interaction of two gene products, termed TAP1 and TAP2. We find that TAPs consisting either of human subunits, or mouse TAP1 and human TAP2, facilitate the presentation of numerous defined viral peptides to mouse class I-restricted T cells. As human and mouse TAP2 and TAP1 differ in 23 and 28% of their residues, respectively, this indicates that TAP1 and TAP2 can form a functional complex with partners considerably different from those they coevolved with. Moreover, these findings indicate that widely disparate TAPs facilitate delivery of the same peptides to class I molecules. These findings suggest that TAP polymorphism does not greatly influence the types of peptides presented to the immune system.

CD8$^+$ T cells (T_{CD8+}) recognize MHC class I molecules bearing antigenic peptides that in most cases derive from a cytosolic pool of proteins (1, 2). Efficient presentation of cytosolic antigens requires the expression of two MHC-encoded proteins (termed TAP1 and TAP2 [transporter associated with antigen processing]) that are members of a family of proteins that transport various molecules across cellular membranes (3–6). In cells lacking expression of one or both functional TAP subunits, the assembly of class I α chains with β_2-microglobulin to form stable class I molecules is compromised (7–10). Since such class I molecules can be stabilized by addition of antigenic peptides, it is believed that TAP functions to transport peptides from the cytosol to the early exocytic compartment where class I assembly normally occurs (7). TAP has not, however, been directly demonstrated to facilitate peptide transport across membranes, and could function in other ways to enhance the delivery of cytosolic peptides to class I molecules (11). TAP genes from humans and rats are polymorphic (12–14). In rats, this polymorphism can directly influence the types of peptides that are bound to class I molecules (12), suggesting that TAP polymorphism might generally greatly influence the types of determinants presented to T_{CD8+}. To address this issue, we examine whether TAPs formed by human TAP1 and TAP2 subunits, or mouse TAP1 and human TAP2 subunits, are capable of facilitating presentation of determinants to mouse T_{CD8+}.

Materials and Methods

Cells and Virus. RMA and RMA/s cells (H-2^b) were provided by Dr. K. Kärre (Karolinska Institute, Stockholm, Sweden). C1R and T2 cells and their K^k-expressing transfectants were provided by Drs. J. Alexander and P. Cresswell (Yale University, New Haven, CT). .45 cells were provided by R. DeMars (University of Wisconsin, Madison, WI). The expression of K^k molecules by transfectants was confirmed by immunoprecipitation of K^k from detergent extracts of [^{35}S]methionine-radiolabeled cells. Cells and transfectants were maintained in RPMI 1640 supplemented with 7.5% (vol/vol) FCS. The influenza virus A/PR/8/34 and the Sendai parainfluenza type I virus were propagated in the allantoic cavity of 10-d-old chicken eggs. The Indiana strain of vesicular stomatitis virus (VSV) was grown in baby hamster kidney cells. Recombinant vaccinia viruses (rVVs) were propagated in thymidine kinase-deficient human 143B osteosarcoma cells. rVVs expressing influenza virus gene products nucleoprotein (NP), basic polymerase 2, and hemagglutinin (HA) without its NH_2-terminal endoplasmic reticulum (ER) insertion sequence (VV-NP, VV-PB2, and VV-L$^-$HA, respectively) and H-2K^d (VV-K^d) have been described (15–17). rVVs expressing H-2 D^d (D^d-VV), or residues 1–168 of the

A/PR/8/34 NP (VV-NP_{1-168}), were produced by inserting cDNAs encoding the respective genes behind the early/late VV p7.5 promoter into a modified pSC11 plasmid as described (18).

Mice. 6–8-wk-old C57BL/6 (H-2^b), CBA/J (H-2^k), and BALB/c (H-2^d) mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Mice were immunized with influenza virus or β-propiolactone-inactivated Sendai virus by intraperitoneal injection, and with rVV by intravenous injection.

Cytotoxicity Assays. Target cells were infected with viruses as described previously (19, 20). T_{CD8+} were generated from splenocytes derived from animals immunized with viruses 2–6 wk previously by 7-d in vitro stimulation with virus-infected autologous splenocytes as described (19, 20) or with synthetic peptides provided by the Biological Resources Branch (NIAID). Microcytotoxicity assays were performed as previously described (19, 20). Data are expressed as percent specific release defined as: 100× [(experimental cpm − spontaneous cpm)/(total cpm − spontaneous cpm)].

Production of Transfected RMA/S Cells. The full-length human TAP2 cDNA (21) was subcloned into RSV.5(neo) plasmid (22) using flanking XbaI sites. RMA/S cells were transfected by electroporation in 0.4-cm cuvettes at 210 V and 960 μFD. Transfectants were selected by growth in 24-well plates in the presence of 1 mg/ml G418. 4 wk after transfection, cells were analyzed by cytofluorography after indirect immunofluorescence using the H-2 D^b-specific mAb 28.14.8S (HB-27; American Type Culture Collection, Rockville, MD). Increased levels of surface staining were detected on 22 of 36 G418-resistant populations. The H-2 K^b-specific mAb Y3 (HB-176; American Type Culture Collection) was also used to quantitate class I surface expression.

Table 1. *Cytofluorographic Analysis of RMA/S Cells Transfected with the Human TAP2 Gene*

Cells	Percent positive		
	Control	Anti-D^b	Anti-K^b
RMA	2 (20)	100 (233)	100 (239)
RMA/S	2 (20)	15 (20)	55 (21)
RMA/S 6.2	1 (20)	95 (49)	98 (70)

Viable cells were incubated with a control mAb specific for VSV G protein, or mAbs specific for D^b (HB-27) or K^b (HB-176). Antibody binding was detected by addition of FITC-conjugated rabbit anti-mouse IgG. Nonviable cells were gated out of analysis based on their light scattering properties. Data are expressed as percent positive (relative to no first antibody) and mean channel fluorescence of positive cells (in parentheses).

Results and Discussion

We first examined whether TAP consisting of mouse TAP1 and human TAP2 could present peptides to mouse T_{CD8+}. RMA/S cells were transfected with a plasmid containing cDNA encoding the human TAP2 and the neomycin resistance gene. RMA/S is a mouse cell line selected from mutagenized RMA lymphoma cells on the basis of reduced class I expression. The single TAP2 gene present in RMA/S cells has a point mutation at nucleotide position 97 resulting in the introduction of a premature stop codon (23). RMA/S cells demonstrate a diminished capacity to present cytosolic antigens to T_{CD8+}, although the severity of the defect varies among antigens (24–26). Antigen processing and class I cell surface expression is enhanced after transfection with mouse or rat TAP2 genes, which indicates that the antigen processing defect is due largely, if not solely, to the absence of normal TAP2 (27, 28).

Two RMA/S transfectant clones resistant to G418 selection expressing increased levels of cell surface D^b by cytofluorography were selected for further study. Additional cytofluorographic analysis of the clones revealed that both demonstrated increased expression of K^b and D^b class I molecules (Table 1). In the tables, results are shown for only the 6.2 clone, which expressed slightly more class I molecules than 5.2. In most of the functional experiments described below, both 5.2 and 6.2 clones were examined, and 5.2 behaved similarly to 6.2. It is notable that although expression of class I molecules was increased by expression of the human TAP2 gene, it remained lower than in RMA cells. While this could be due to differences between human and mouse TAP2 subunits that limit the effectiveness of human TAP2 in mouse cells, a similar difference was noted between RMA cells and RMA/S cells transfected with the mouse TAP2 gene (27). As noted previously (27), RMA/s cells may be defective in other genes that enhance class I assembly, transport, or stability.

The ability of clone 6.2 to present cytosolic antigens from influenza or Sendai viruses to T_{CD8+} was investigated next. T_{CD8+} specific for influenza virus NP were generated by PR8 in vitro stimulation of splenocytes derived from mice primed with a rVV-expressing NP. Such T_{CD8+} have been shown to recognize a single D^b peptide from NP corresponding to residues 366–374 (28a). T_{CD8+} specific for Sendai virus NP were generated by Sendai virus stimulation of splenocytes derived from Sendai virus–primed mice. Based on the findings of Kast et al. (29), it is likely that these T_{CD8+} principally recognize a single K^b-restricted peptide corresponding to NP residues 321–328. As seen in Table 2, 6.2 cells demonstrated enhanced presentation of both viral NPs.

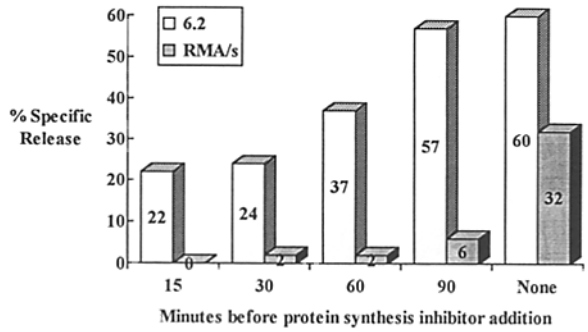
The antigen processing capacity of 6.2 cells was further characterized using T_{CD8+} specific for a peptide corresponding to residues 52–59 from VSV N (30). After infection with VSV, 6.2 cells were lysed at higher levels than RMA/S cells (Fig. 1). Since cytotoxicity assays provide a poor quantitative measure of the amount of peptide–class I complexes displayed on the cell surface, the recognition of cells expressing limiting amounts of peptides was tested. Cells were treated at various times after VSV infection with either a mixture of protein synthesis inhibitors or the fungal metabolite, brefeldin A (BFA), which blocks the exocytosis of peptide–class I complexes formed in the ER (31, 32). These treatments provide, respectively, measures of the efficiency of peptide generation from a limited amount of protein, and the rate at which class I–peptide complexes are delivered to the cell surface in the absence of protein synthesis inhibitors. Addition of protein synthesis inhibitors to RMA/S cells greatly

Table 2. *Presentation of Individual Influenza and Sendai Virus Antigens by Transfected RMA/S Cells Expressing the Human TAP2 Gene*

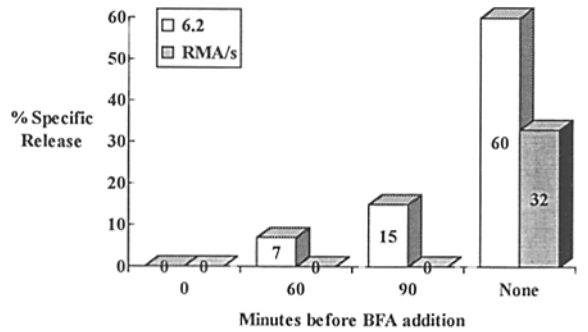
		Percent specific ^{51}Cr release			
		Antiinfluenza NP		Anti-Sendai	
Exp.	Cells	10:1	5:1	40:1	20:1
A	RMA	26	6		
	RMA PR8	85	70		
	RMA/S	5	0		
	RMA/S PR8	31	9		
	RMA/S 6.2	4	2		
	RMA/S 6.2 PR8	65	56		
B	RMA			3	3
	RMA Sendai			85	72
	RMA/S			2	3
	RMA/S Sendai			31	15
	RMA/S 6.2			5	4
	RMA/S 6.2 Sendai			83	80

Uninfected or virus-infected cells were tested in a 4-h ^{51}Cr release assay at the E/T ratios indicated. T_{CD8+} specific for individual influenza virus antigens were generated by in vitro PR8 stimulation of splenocytes derived from mice immunized with VV-NP. T_{CD8+} specific for Sendai virus were generated by in vitro Sendai virus stimulation of splenocytes derived from mice immunized with Sendai virus.

A



B



compromised their presentation of antigen to T_{CD8+} (Fig. 1 *A*). By contrast, presentation by 6.2 cells was less affected, and control values of lysis were reached by allowing protein synthesis to proceed for 90 min after infection. Indeed, the residual and constant levels of lysis observed at the 0–30-min time points with 6.2 cells probably represents breakthrough biosynthesis of nucleocapsid (N) protein at levels too low to sensitize nontransfected RMA/S cells. Similarly, while BFA completely blocked presentation of N by RMA/S cells even when added as late as 90 min after infection, 6.2 cells transported sufficient peptide–class I complexes to enable recognition of some cells as soon as 60 min after infection (Fig. 1 *B*). This is similar to the kinetics of presentation of N by RMA cells (24). Thus, we conclude that expression of the human TAP2 gene concomitantly enhances the efficiency of RMA/S cells to produce antigenic peptides from a limited pool of protein, and increases the rate at which class I–peptide complexes can be produced and transported to the cell surface.

Figure 1. Effect of inhibitors on presentation of VSV N by RMA/S and 6.2 cells. (*A*) Cycloheximide (15 μg/ml) and anisomycin (26 μg/ml) were added to cells at the times indicated after infection and maintained throughout the infection and ^{51}Cr labeling periods until the 4-h microcytotoxicity assay, in which only cycloheximide (15 μg/ml) was present. (*B*) BFA (5 μg/ml) was added to cells at the times indicated after infection and maintained throughout the infection and ^{51}Cr labeling periods until the 4-h microcytotoxicity assay, in which BFA was present at 1.25 μg/ml. BFA or cycloheximide at these concentrations do not inhibit T_{CD8+} activity (31). The actual percent specific release values are given within the bar depicting the time point.

Table 3. *TAP-dependent Presentation of Mouse Class I-restricted Peptides by Human Cells*

Exp.	Cells	Percent specific release					
		K^{k*} HA‡ 245–252§ (18:1)	K^k HA 340–348 (4:1)	K^k NP 50–57 (6:1)	K^d HA 519–527 (3:1)	K^d NP 147–155 (20:1)	D^d PR8 (20:1)
A	C1R K^k + VV	10	3	7			
	C1R K^k + VV L⁻HA	49	37				
	C1R K^k + VV-NP_{1-168}			62			
	T2 K^k + VV	5	1	4			
	T2 K^k + VV L⁻HA	5	5				
	T2 K^k + VV-NP_{1-168}			6			
B	C1R + VV-K^d				1	2	
	C1R + VV-K^d + VV-L⁻HA				41		
	C1R + VV-NP					50	
	T2 + VV-K^d				0	3	
	T2 + VV-K^d + VV-L⁻HA				8		
	T2 + VV-K^d + VV-NP					3	
C	.45 + VV-D^d						0
	.45 + VV-D^d + VV-PB2						42
	T2 + VV-D^d + VV-PB2						0

Uninfected or virus-infected cells were tested in a 6-h ^{51}Cr release assay at the E/T ratios indicated. Presentation of PB2 was assessed using T_{CD8+} induced by influenza virus in vitro stimulation of splenocytes derived from influenza virus–infected mice. The other T_{CD8+} were generated from spleneocytes derived from mice immunized with rVVs expressing individual influenza virus proteins by in vitro stimulation with the synthetic peptide corresponding to the naturally processed determinant. Indirect immunoperoxidase staining using mAbs specific for VV gene products demonstrate that T2 cells expressed equal or greater amounts of VV-encoded proteins than .45 or C1R cells. Additionally, K^k-transfected T2 cells and VV K^d-infected T2 cells are able to efficiently present determinants delivered to the ER through the action of a NH_2-terminal leader/signal peptide (39, and our unpublished results).

* Restriction element.

‡ Specificity of T_{CD8+}.

§ Peptide recognized.

These findings demonstrate that human TAP2 can substitute for its mouse homologue in facilitating the presentation of each of three cytosolic viral antigens tested. If TAP1 and TAP2 function as a complex as believed, this would mean that widely different subunits can be substituted for each other without grossly affecting function. This implies that the human TAP functions similarly to mouse TAP.

To test this idea, we examined the capacity of EBV-transformed human B cell lines (C1R and .45) expressing mouse class I molecules to present peptides to mouse T_{CD8+}. Mouse class I molecules were expressed from either transfected genes (K^k) or genes inserted into rVVs (K^d and D^d). In either case, influenza virus proteins were expressed by infection with rVVs expressing the relevant influenza virus protein. Mouse T_{CD8+} specific for each of five defined peptide determinants tested from influenza virus HA and NP (33–35) were able to lyse human cells expressing the appropriate class I molecules and viral protein (Table 3). Additionally, an undefined D^d-restricted determinant from PB2 (36) was presented to polyclonal anti-influenza virus mouse T_{CD8+}. To establish the TAP dependence of antigen presentation, antigen processing–deficient T2 cells (10, 37) were included in each of the experiments. These cells possess a single copy of chromosome 6 that contains a 1-Mbp deletion in the HLA region encoding the TAP. Despite expressing similar amounts of VV-encoded proteins (not shown), T2 cells were lysed at or near background levels by each of T_{CD8+} tested. In additional experiments, we found that the presentation of each of these antigens can be reconstituted by the expression of TAP1 and TAP2 genes in the absence of other MHC gene products (our unpublished results). Similar TAP-dependent presentation was found for three defined K^b-restricted determinants from Sendai NP, VSV N, and OVA (38, and not shown), and for undefined VV-derived determinants in association with K^b, K^k, D^b, K^d, and L^d (not shown). Thus, all told, human TAP was able to facilitate the presentation of each of at least 14 viral determinants examined.

Our findings indicate that human and mouse TAPs func-

tion to facilitate presentation of the same set of peptides. As these TAPs are almost certain to exhibit greater differences than TAPs within a given species (human and mouse TAP2 and TAP1 differ, respectively, in 23 and 28% of their residues), this suggests that polymorphism in TAPs does not greatly affect the repertoire of peptides presented by class I molecules to the immune system. The recent findings of Lobigs and Müllbacher (40) lead to the same conclusion. This conclusion conflicts with the observation of the relatively large effect observed on peptides derived from rat RT1.A^a class I molecule after the expression of a rat TAP2 allele that differed from the naturally coexpressed allele by as few as 25 residues out of 703 (4% nonhomology) (12). There are a number of potential explanations for this apparent discrepancy. (*a*) Differences in TAP peptide specificity are of a more quantitative than qualitative nature, and such differences are difficult to detect by the measure of T_{CD8+} recognition. (*b*) Rat TAP2 is particularly sensitive to amino acid alterations. (*c*) TAP2 genes can accommodate a large number of changes in many positions without altering its peptide specificity (or changes in different locations balance the effect of one another), while particular changes in certain residues have a large effect on peptide specificity. (*d*) Amino acid substitutions in TAP2 affect the interaction of the TAP complex with only a subset of peptides, such a subset being preferentially bound by RT1.A^a molecules.

Distinguishing among these possibilities will require further efforts. Ultimately, to establish the effect of TAP polymorphism on T_{CD8+} responses in vivo, it will be necessary to produce transgenic animals expressing foreign TAP alone, or in combination with their natural TAP.

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