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TAP1-Dependent Peptide Translocation In Vitro Is ATP Dependent and Peptide Selective

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Summary

T cells detect infection of cells by recognizing peptide fragments of foreign proteins bound to class I molecules of the major histocompatibility complex (MHC) on the surface of the infected cell. MHC class I molecules bind peptide in the endoplasmic reticulum, and analysis of mutant cells has demonstrated that an adequate supply of peptides requires the presence of two genes in the MHC class II locus that encode proteins called transporters associated with antigen processing (TAP) 1 and 2. TAP1 and TAP2 are members of the ATP-binding cassette family of membrane translocators. In this study, we demonstrate in a cell-free system that TAP1 is part of an ATP-dependent, sequence-specific, peptide translocator.

Introduction

Antigen processing for presentation by major histocompatibility (MHC) class I molecules involves proteolysis of intracellular polypeptide antigens and supply of peptides to the site of class I protein assembly, in the lumen of the rough endoplasmic reticulum. Class I molecules complexed with peptide are transported to the cell surface, where they can be recognized by the appropriate CD8⁺ T cell receptor (Neefjes et al., 1991).

Recently, two genes, designated *TAP1* and *TAP2* (TAP: transporter associated with antigen processing), have been cloned from the MHC region of several species (Deverson et al., 1990; Monaco et al., 1990; Spies et al., 1990; Trowsdale et al., 1990). The deduced amino acid sequences of these genes have led to the suggestion that they might function in antigen processing and presentation. The TAP proteins are members of the ATP-binding cassette (ABC) family of membrane translocators, which includes the cystic fibrosis chloride channel (Riordan et al., 1989), the yeast STE6 protein (McGrath and Varshavsky, 1989), the multidrug resistance p-glycoprotein (Gros et al., 1986), and the Salmonella typhimurium oligopeptide transporter (Hiles et al., 1987). These proteins all have a hydrophobic domain that is thought to span the membrane six to eight times and a cytosolic nucleotide-binding domain. Both domains are repeated twice, either on a single polypeptide or by dimerization of separate polypeptides (Hyde et al., 1990; Blight and Holland, 1990). The different ABC translocators actively translocate their substrates across membranes.

Based on the sequence homology of TAP1 and TAP2 to other ABC translocators and the proposed localization of TAP1 in the endoplasmic reticulum (Kleijmeer et al., 1992), it has been hypothesized that the TAP proteins pump peptides proteolytically derived from antigens in the cytosol into the lumen of the endoplasmic reticulum (Deverson et al., 1990; Monaco et al., 1990; Spies et al., 1990; Trowsdale et al., 1990). These peptides are subsequently assembled into a complex with β2-microglobulin and class I heavy chain (Townsend et al., 1989). In keeping with this hypothesis, two mutant cell lines, RMA-S from mouse (Ljunggren and Karre, 1985) and 721.174 from human (DeMars et al., 1984), have mutations or deletions in the MHC genes, which include *TAP2* in RMA-S (Powis et al., 1991; Attaya et al., 1992) and *TAP1* and *TAP2* in 721.174 (Spies et al., 1990; Spies and DeMars, 1991). In the RMA-S cell line labile class I molecules that lack peptide are delivered to the surface (Ljunggren et al., 1990; Ortiz-Navarette and Hammerling, 1991). Addition of presentable peptide stabilizes class I molecules at the cell surface (Townsend et al., 1989, 1990; Ljunggren et al., 1990; Schumacher et al., 1990). Thus, peptide supply to class I molecules is deficient in these mutant cell lines. Transfection of the RMA-S cell line with the *TAP2* gene (Powis et al., 1991; Attaya et al., 1992) or of the 721.174 cell line with both *TAP1* and *TAP2* genes (Momburg et al., 1992) restored stable surface class I expression. These results suggest that the relevant molecular species is a complex of TAP1 and TAP2, and indeed, coimmunoprecipitation of the two proteins supports this hypothesis (Kelly et al., 1992; Spies et al., 1992).

Mice deficient in TAP1 (Van Kaer et al., 1992) lack stable surface H-2K^b and H-2D^b molecules, but addition of peptide to spleen cells derived from the mouse stabilizes empty class I molecules at the cell surface. There is a major reduction in terminally glycosylated class I molecules in the mutant mouse, indicating that empty, unstable class I molecules accumulate in the endoplasmic reticulum. Thus, the *TAP1* gene is essential in vivo for expression of stable class I molecules on the surface of cells, probably through its role in peptide supply. However, to date there is no direct evidence that these molecules actually transport peptides nor that such transport is ATP dependent. Indeed, three studies show peptide translocation in the absence of ATP (Lévy et al., 1991a, 1991b; Koppelman et al., 1992; Bijlmakers et al., 1993).

In this report, we describe an in vitro assay that measures translocation of a radiolabeled 9 amino acid peptide into the lumen of crude microsomes purified from *TAP1−/−* and *TAP1+/+* mice. The assay reveals that the TAP1 protein is required for ATP-dependent peptide trans-

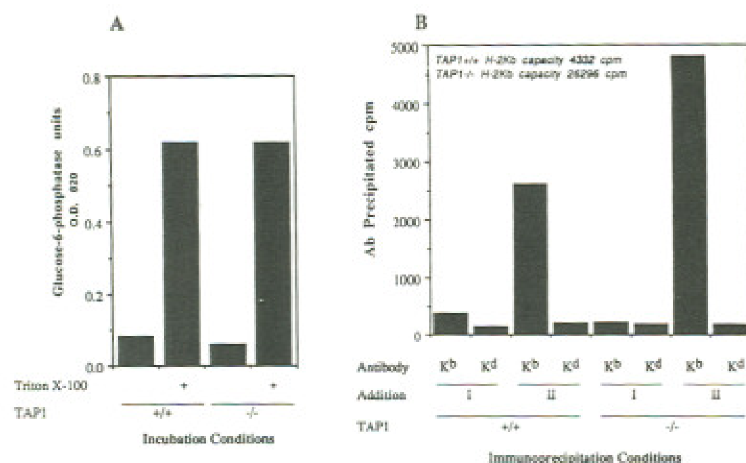

Figure 1. Both *TAP1+/+* and *TAP1−/−* Microsomes Are Intact and Can Internalize Peptide That Binds to H-2K^b

(A) Microsomes were assayed in duplicate for glucose 6-phosphatase for 15 min at 37°C in the presence or absence of 0.1% Triton X-100 by the method of Aronson and Touster (1974).
(B) Microsomes were incubated in duplicate with 0.5 μM labeled FAPGNYPAL and 50 μM ATP in a regenerating mix for 1 hr at 37°C and then incubated with Y3 (αK^b) or SF1-1.1.1 (αK^d) for 1 hr on ice. The microsomes were then recovered through sucrose cushions and lysed, and either Gamma G beads were added (I) or a further 1 hr incubation with antibody on ice was performed (II), and then Gamma G beads were added. The italicized insert represents cpm immunoprecipitated by Y-3 after 1% NP-40-lysed microsomes were incubated with 0.5 μM labeled FAPGNYPAL for 1 hr at 37°C.

location into the lumen of the microsomes. The translocated peptides bind to class I H-2K^b molecules within the microsomes. This assay has allowed us to demonstrate that the TAP1-dependent translocator has selectivity for the sequence of peptides and may translocate peptides longer than those usually found associated with class I molecules.

Results

Assay for Peptide Transport into Microsomes

To establish an in vitro assay for peptide transport into the endoplasmic reticulum, microsomes were prepared from *TAP1+/+* and *TAP1−/−* mice. For the assay to be valid, the microsomes must be intact. A lumenal enzyme of the rough endoplasmic reticulum, glucose 6-phosphatase, was inaccessible to a membrane-impermeable substrate added to the cytosolic side of the membrane (Figure 1A). Addition of detergent to the microsomes indicated that at least 90% of the microsomes remained intact during a 15 min incubation at 37°C. The activity of glucose 6-phosphatase in the presence of detergent was used to standardize the amount of microsomes added to a translocation assay.

As a further measure of the integrity of the microsomes, they were assessed for their ability to assemble complexes of lumenal H-2K^b with the radiolabeled H-2K^b-binding Sendai virus nucleoprotein peptide 324–332 FAPGNYPAL (Schumacher et al., 1991). After 1 hr at 37°C, H-2K^b molecules in both *TAP1+/+* and *TAP1−/−* microsomes were able to assemble with radiolabeled FAPGNYPAL (Figure 1B). As truncated versions of FAPGNYPAL are unlikely to bind to H-2K^b in a stable fashion (Deres et al., 1992), intact peptide was likely to be supplied to H-2K^b in both populations of microsomes.

To determine whether the H-2K^b–peptide complexes were assembled lumenally or whether H-2K^b-binding sites for peptide were directly exposed to the incubation medium, microsomes already loaded with radiolabeled peptide were incubated, before or after solubilization, with anti-K^b or anti-K^d antibody (Figure 1B). Incubation of microsomes with antibody before solubilization, followed by lysis and immunoprecipitation (I), showed that close to all of H-2K^b in *TAP1−/−* microsomes was inaccessible to antibody before solubilization and >90% in *TAP1+/+* microsomes, as compared with incubation of microsomes with

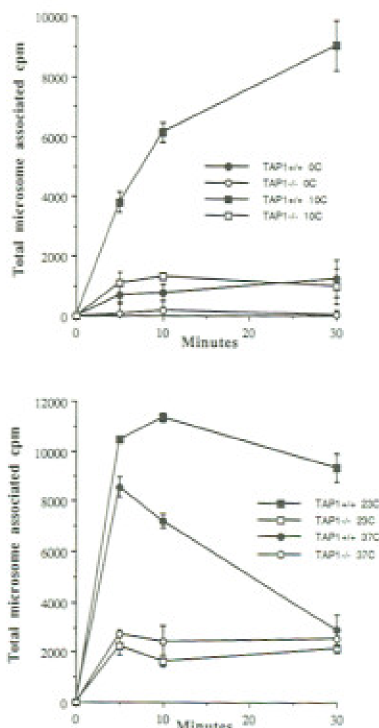

Figure 2. *TAP1+/+* Microsomes Accumulate Peptide at a Higher Rate Than *TAP1−/−* Microsomes

Assays containing 50 μM ATP in a regenerating mix and 0.5 μM FAPGNYPAL were assembled in duplicate on ice and incubated at 0°C, 10°C, 23°C, or 37°C for 0, 5, 10, or 30 min and then pipetted onto the surface of ice-cold sucrose cushions containing 10 μM cold FAPGNYPAL, and the microsomes were pelletted. The lysed pellets were counted. Similar results were obtained using several independently prepared batches of microsomes.

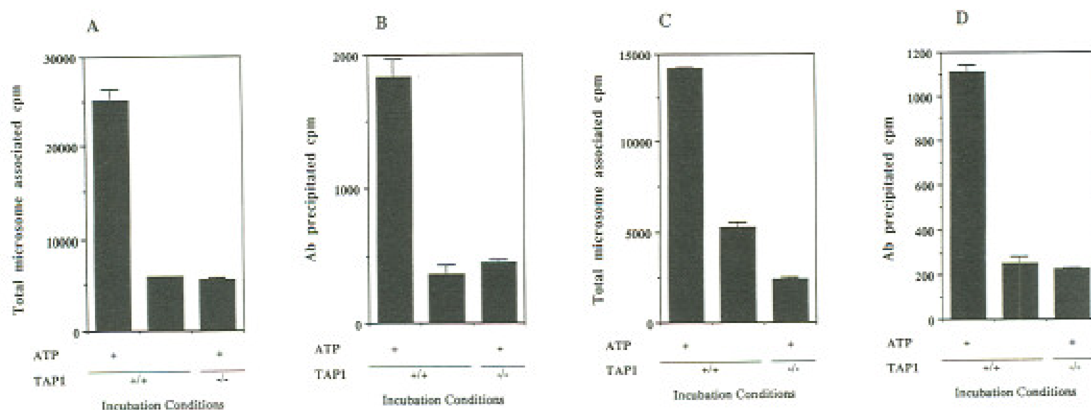

Figure 3. TAP1-Dependent Translocation of FAPGNYPAL Requires ATP

Assays were incubated in duplicate for 10 min at 23°C with 0.5 μM labeled FAPGNYPAL and an ATP-regenerating mix containing 50 μM ATP or no ATP. Microsomes were then collected through sucrose cushions containing 10 μM unlabeled FAPGNYPAL (A and B) or, in a separate experiment, through cushions containing no unlabeled FAPGNYPAL (C and D), and an aliquot of the lysed pellet was counted (A and C). For (B) and (D), an aliquot of the lysed pellet was incubated with Y3 (αK^b) or SF1-1.1.1 (αK^d) and immunoprecipitated. Background SF1-1.1.1-precipitated cpm were subtracted from Y3-precipitated cpm. In separate experiments it was shown that peptide accumulation by *TAP1−/−* microsomes was ATP independent. Similar results were obtained using several independently prepared batches of microsomes, duplicates + SD.

antibody before and after solubilization (II). The low but significant level (<10%) of H-2K^b complexed with peptide that was accessible to antibody before membrane solubilization in *TAP1+/+* microsomes suggests that either the *TAP1+/+* microsomes are more labile than *TAP1−/−* microsomes, contrary to the glucose 6-phosphatase data, or that the *TAP1+/+* microsomes contain some 10% of "inside-out" vesicles. Similar results to those presented in Figure 1B are obtained when peptide is loaded for 1 hr at 37°C in the presence or absence of ATP (data not shown), indicating that ATP is not required for supply of peptide or assembly of H-2K^b with peptide in either *TAP1+/+* or *TAP1−/−* microsomes incubated for this time and temperature.

In detergent-solubilized microsomes incubated with radiolabeled peptide for 1 hr at 37°C, there was approximately 6 times as much empty H-2K^b available for peptide binding in the *TAP1−/−* microsomes compared with *TAP1+/+* microsomes (Figure 1B, italicized insert). Presumably, the peptide concentration in the lumen of the endoplasmic reticulum is much lower in vivo in *TAP1−/−* mice, resulting in an increased level of empty H-2K^b molecules (Van Kaer et al., 1992).

TAP1+/+ Microsomes Accumulate Peptide at a Higher Rate Than *TAP1−/−* Microsomes

As the experiments described above indicated that both *TAP1+/+* and *TAP1−/−* microsomes are capable of loading H-2K^b with peptide after 1 hr at 37°C, we investigated whether TAP1 influences the rate of peptide accumulation by microsomes. Figure 2 shows the accumulation of peptide with time by microsomes at different temperatures. At 23°C *TAP1+/+* microsomes accumulated peptide to an apparent maximum in 10 min or less, and then no further accumulation occurred. At 37°C the maximum accumulation of peptide by *TAP1+/+* microsomes was reached in under 5 min and then an apparent efflux of peptide, or of degradation products of peptide, occurred over the subsequent 25 min of incubation. At 10°C the initial rate of peptide accumulation by *TAP1+/+* microsomes could be measured for periods of less than 10 min and was found to be 4 times higher than *TAP1−/−* peptide accumulation (Figure 2). Efflux of peptide is less apparent at 10°C. The amount of radiolabeled peptide accumulated by *TAP1+/+* microsomes is far in excess of H-2K^b-binding sites, indicating that peptide accumulation is not coupled to H-2K^b–peptide binding.

ATP Is Required for TAP1-Dependent Peptide Internalization

The experiments presented in Figure 3A demonstrate a requirement for ATP for the accumulation of FAPGNYPAL into *TAP1+/+* microsomes. In the absence of ATP, *TAP1+/+* microsomes accumulated the same amount of FAPGNYPAL as *TAP1−/−* microsomes, whereas in the presence of 50 μM ATP and an ATP-regenerating system, *TAP1+/+* microsomes accumulated about 4 times as much peptide. When transport assays were carried out in the absence of an ATP-regenerating system, ATP (1 mM) but not ATPγS (1 mM) could drive accumulation (data not shown). This is consistent with a requirement for ATP hydrolysis for peptide uptake. Omission of an ATP-regenerating system prevented any contaminating ADP in the ATPγS from driving peptide accumulation. Only in the presence of ATP do the *TAP1+/+* microsomes transport added peptide in a TAP-dependent fashion, as shown by solubilization of microsomes and immunoprecipitation of H-2K^b (Figure 3B).

In our standard assay, unlabeled FAPGNYPAL is included in the sucrose cushion through which the microsomes are recovered to block free peptide-binding sites during pelletting. When in a separate experiment unlabeled FAPGNYPAL was omitted from the sucrose cushion (Figures 3C and 3D), a 2-fold increase in peptide accumu-

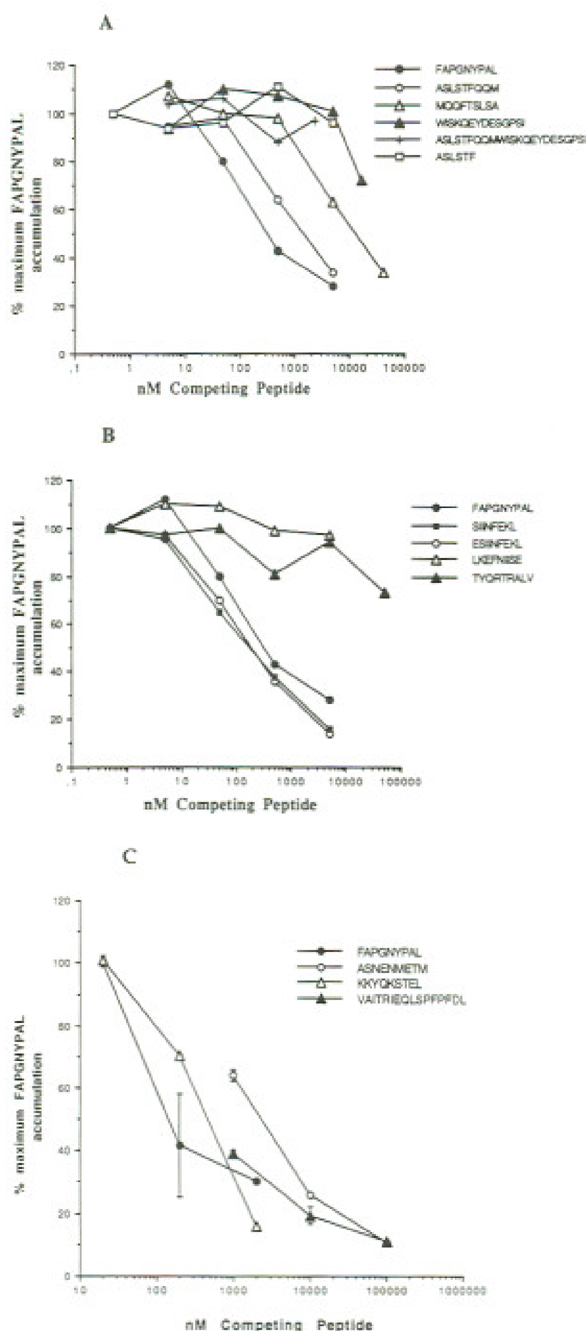

Figure 4. Competition for *TAP1+/+* FAPGNYPAL Accumulation Is Peptide Selective

(A and B) Unlabeled competing peptides were titrated by 10-fold dilutions into assays containing 0.5 μM labeled FAPGNYPAL, ATP, and *TAP1+/+* microsomes. Labeled and unlabeled peptide were added simultaneously, assays were incubated for 10 min at 23°C, and the microsomes were pelletted through sucrose cushions, lysed, and counted. Maximum FAPGNYPAL accumulation was determined for each peptide titration.

(C) Unlabeled peptides were tested in competition assays using 0.2 μM ^{3}H-FAPGNYPAL, an ATP/ATP-regenerating system, and *TAP+/+* microsomes under similar conditions as in (A) and (B). Maximum and ATP-independent FAPGNYPAL accumulation was determined for each peptide titration.

Values given are means of duplicates ± SD.

lated by *TAP1+/+* microsomes over *TAP1−/−* microsomes was observed in the absence of ATP (Figure 3C). This peptide was not internalized since a proportional increase in peptide bound to H-2K^b was not seen when ATP was absent and unlabeled FAPGNYPAL was left out of the sucrose cushion (Figure 3D). This fraction of accumulated peptide can presumably be competed off TAP1/TAP2 by unlabeled peptide (compare Figures 3A and 3C). Thus, the peptide associated with *TAP1+/+* microsomes in the absence of ATP is not made available to the lumenal H-2K^b molecules but may bind to the TAP1/TAP2 translocator.

Selectivity of TAP1-Dependent Peptide Uptake

To test whether the TAP1-dependent accumulation of peptide shows selectivity for peptides, synthetic peptides were added to microsomes at different concentrations to compete for uptake with labeled FAPGNYPAL (Figure 4 and Table 1). The competition observed was for TAP1-dependent accumulation, as inclusion of competing peptide in accumulation assays using *TAP1−/−* microsomes required a concentration of approximately 5 μM of all the peptides to reduce FAPGNYPAL uptake, regardless of sequence or length of the peptide (data not shown). In addition, the TAP1-independent accumulation of peptide was only 20% of the total peptide accumulated by *TAP1+/+* microsomes and could not account for the extent of inhibition observed. The peptides were derived from sequences of peptides eluted from H-2K^b by one of us (S. I. et al., submitted) or previously shown to bind to H-2K^b, H-2K^d, H-2D^b, H-2L^d, or HLA-B27 by others (Rotzschke et al., 1990; Falk et al., 1991; Udaka et al., 1992; J. Strominger, personal communication). Figure 4 shows the accumulation of labeled FAPGNYPAL by *TAP1+/+* microsomes in the presence of dilutions of unlabeled peptides. The arbitrary figure of 50% uptake was chosen to determine the competing peptide concentrations presented in Tables 1A and 1B. The relative inhibitory potencies calculated in Tables 1A and 1B show that some peptides are better substrates, some are a little worse, and some are much worse than FAPGNYPAL. The concentrations required for inhibition by the best and worst substrates differed by a factor of at least 250.

The H-2D^b-restricted influenza virus nucleoprotein peptide ASNENMETM blocks uptake of labeled FAPGNYPAL (Figure 4C and Table 1B), although this peptide does not bind to H-2K^b (Van Kaer et al., 1992). Thus, the observed competition between peptides is not related to their ability to compete with FAPGNYPAL for binding to empty H-2K^b molecules. The optimal substrate derived from proteolysis of the actin molecule appears to be between 7 and 13 amino acids. However, a 16 amino acid peptide, VAITRIEQLSPFPFDL, competes for accumulation with an efficiency similar to that of 8 or 9 amino acid peptides (Table 1B). Based on the concentrations of competitor peptide required to observe 50% inhibition of uptake, the K_m for peptide of the TAP translocator is likely to be in the submicromolar range (Figure 4; Table 1). In two instances, ESIINFEKL and ASLSTFQQM, the peptides made by reversing the amino acid sequences were much poorer

Table 1. Summary of the Inhibitory Potencies of Different Peptides for TAP1-Dependent FAPGNYPAL Translocation

Competitor	Source	Restriction	IC_{50} (μM)	IC_{50} Competitor / IC_{50} FAPGNYPAL
A. FAPGNYPAL	$^{324-332}$SeV NP	K^b	0.35	1
ASLSTF	$^{347-352}$Actin	K^b	>5.0	>14.3
ASLSTFQQM	$^{347-355}$Actin	K^b	1.5	4.3
MQQFTSLSA			15	43
WISKQEYDESGPSI	$^{356-369}$Actin	K^b	50	143
ASLSTFQQMWISKQEYDESGPSI	$^{347-369}$Actin	K^b	>2.3	>6.6
SIINFEKL	$^{257-264}$Ovalbumin	K^b	0.2	0.6
ESIINFEKL	$^{256-264}$Ovalbumin	K^b	0.2	0.6
LKEFNIISE			5.0	>14.3
TYQRTRALV	$^{147-154}$IFV NP	K^d	>50	>143
B. FAPGNYPAL	$^{324-332}$SeV NP	K^b	0.15	1
ASNENMETM	$^{366-374}$IFV NP	D^b	1.8	12
VAITRIEQLSPFPFDL	Unknown	L^d	0.45	3
KKYQKSTEL	Unknown	HLA-B27	0.45	3

The concentration of competing peptide inhibiting 50% of labeled FAPGNYPAL accumulation was determined and expressed as relative inhibitory potency (IC_{50} competitor/IC_{50} FAPGNYPAL). SeV NP, Sendai virus nucleoprotein; IFV NP, influenza virus nucleoprotein. Residues are numbered N- to C-terminus as found in nature.

(A) [^{3}H-FAPGNYPAL] = 0.5 μM. All natural peptides are H-2K^b restricted except IFV NP 147–154, which is H-2K^d restricted (Falk et al., 1991; S. I. et al., submitted).

(B) [^{3}H-FAPGNYPAL] = 0.2 μM. The peptide sequence ASNENMETM is from Rotzschke et al., 1990. The peptide sequence VAITRIEQLSPFPFDL is from Udaka et al., 1992. The underlined sequence binds H-2L^d. The peptide sequence KKYQKSTEL is derived from a peptide eluted from HLA-B27 (J. Strominger, personal communication).

substrates for TAP1-dependent uptake (Table 1A). The reversed sequence actin peptide competed at a 10-fold higher concentration for TAP1-dependent uptake of peptide compared with the natural peptide, whereas the reversed sequence ovalbumin peptide did not compete for TAP1-dependent transport up to the limit of its solubility, a factor of at least 25-fold. Thus, for these two peptides, sequence determines affinity for TAP1-dependent translocation and not overall charge or hydrophobicity. Interestingly, the substrate with the lowest affinity for TAP1-dependent translocation is a 9 amino acid peptide from influenza virus nucleoprotein that is H-2K^d restricted (Falk et al., 1991).

Discussion

The in vitro experiments presented here demonstrate that TAP1 functions to increase the rate of peptide translocation across microsomal membranes in an ATP-dependent fashion. This function is essential in vivo for cell surface expression of MHC class I molecules and positive selection of T cells in the thymus (Van Kaer et al., 1992; Ashton-Rickardt et al., 1993). Earlier reports of peptide translocation across microsomal membranes found that peptides could translocate in the absence of ATP (Koppelman et al., 1991; Lévy et al., 1991b; Bijlmakers et al., 1993) and in the absence of the human homologs of the TAP proteins (Lévy et al., 1991b). However, our studies indicate that the concentration of added peptide, and the temperature and length of incubation employed in the earlier studies, should allow peptide to cross the endoplasmic reticulum bilayer in an ATP-independent manner. The in vitro assay for TAP function described here reveals that the TAP mechanism accumulates peptide rapidly to a plateau level and can only be measured accurately at low temperatures for short incubations. The inability to demonstrate TAP dependency in vitro at 37°C is a shortcoming of the present approach, and further studies are needed to resolve this. However, at reduced temperature TAP and ATP dependencies of peptide transport have now been established. The pathway allowing TAP-independent translocation of peptides is not understood, but is likely to require higher cytosolic concentrations of peptide than TAP1-dependent translocation (Zweerink et al., 1993).

Our results suggest that ATP is not required for peptide binding to a TAP1-dependent structure on the cytosolic face of microsomal membranes (this structure is presumably TAP1 itself or a TAP1–TAP2 heterodimer [Kelly et al., 1992; Spies et al., 1992]). However, no uptake occurs in the absence of added ATP, and ATP hydrolysis seems to be required for translocation. If the ATP-binding site of the TAP translocator is cytosolically disposed, as indicated by comparisons of the TAP sequence with other ABC translocators (Monaco et al., 1990), then TAP1-dependent peptide translocation is likely to be vectorial and another translocation mechanism is presumably responsible for the peptide efflux at 37°C. Efflux of peptide in this system is ATP dependent and temperature dependent (our unpublished data), suggesting that this is an active process.

A characteristic of TAP1-dependent translocation described in this study is its selectivity. A sequence-dependent difference in peptide translocation was observed before (Koppelman et al., 1992); however, its significance is unclear because the translocation measured in the previous study was ATP independent and the involvement of TAP could not be assessed. The selectivity

of the translocator observed in our assay is different from the selectivity of the class I molecule itself, since significant differences exist between the inhibitory potency of peptides for translocation and their affinities for the H-2K^b molecule. However, the conclusions drawn from such comparisons must be qualified by the consideration that certain peptides tested may undergo proteolytic cleavage in the lumen of the endoplasmic reticulum different from the original. The H-2D^b-restricted peptide, ASNENMETM, and the HLA-B27-restricted peptide, KKYQKSTEL, although lacking the H-2K^b motif, are both effective competitors for translocation. The ovalbumin peptides SIINFEKL and ESIINFEKL are equivalent substrates for translocation, but ESIINFEKL has a 50-fold lower affinity for H-2K^b than does SIINFEKL (Imaeda et al., 1993). The actin peptide, ASLSTFQQM, has a 3600-fold lower affinity for H-2K^b than SIINFEKL (Imaeda et al., 1993), although its inhibitory potency for TAP1-dependent peptide accumulation is only 7.5-fold less than that of SIINFEKL.

The low molecular mass polypeptide (LMP)–containing proteosome (Brown et al., 1991; Glynne et al., 1991; Kelly et al., 1991; Martinez and Monaco, 1991; Ortiz-Navarette and Hammerling, 1991) is likely to be involved in cytosolic processing and may be responsible for producing peptides with a high affinity for the TAP translocator. In this study sequence specificity has been demonstrated for the H-2K^b-restricted 8 or 9 amino acid peptides presented to the TAP translocator. This is best illustrated by the vast difference in inhibitory potency of the ovalbumin- and actin-derived peptides versus their reversed sequence analogs. Our results also show that a longer, 16 amino acid peptide can compete effectively for the TAP1 translocator. If this peptide is translocated, rather than simply bound to the cytosolic face of the translocator, then it may be further proteolytically processed in the lumen of the endoplasmic reticulum either before or after binding a class I molecule (Falk et al., 1990). In addition, this 16 amino acid precursor of an H-2L^d-binding peptide, a peptide that binds to human HLA-B27 molecules, also competes for mouse b haplotype TAP translocation, suggesting that TAP polymorphism may not grossly influence the type of peptides supplied to class I molecules. This is in accordance with two recent reports of the lack of influence of different *TAP* alleles on peptides presented by class I molecules (Lobigs and Mullbacher, 1993; Yewdell et al., 1993). Interestingly, a 9 amino acid peptide, TYQRTRALV, presented by H-2^d haplotype mice but not by H-2^b haplotype mice, is among the poorest competitors for translocation. The failure of this peptide to compete for uptake in our assay could be due to its precursor peptide, rather than the peptide itself, being the preferred substrate for translocation. Alternatively, the d haplotype TAP translocator may have a higher affinity for TYQRTRALV than does the b haplotype TAP translocator. In rat there is evidence that TAP translocator polymorphism may affect the supply of certain sets of peptides to class I molecules (Powis et al., 1992). The assay described here should allow a systematic exploration of the specificity of TAP translocators from a variety of sources.

Experimental Procedures

Preparation of Microsomes

Six- to eight-week-old C57BL/6 mice (*TAP1+/+*) or (129/Sv × C57BL/6)F2 (*TAP1−/−*) mice were injected intraperitoneally with 0.2 mg of polyinosinic–polycytidylic acid (Sigma) per mouse 24–36 hr before sacrifice by CO_2. The liver, spleen, and thymus were immediately removed from each mouse and collected in a beaker on ice. All subsequent steps were performed on ice. The organs were washed repeatedly in 50 mM triethanolamine, 50 mM KOAc (pH 7.5), 250 mM sucrose, 6 mM MgOAc, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 0.027 U/ml aprotinin, 1 mM EDTA (pH 7.5) (buffer A) to remove blood and then weighed. Approximately 9 g of tissue was obtained from ten mice. Buffer A was added (4 ml per gram of tissue), and the tissue was then homogenized in a motor-driven Potter–Elvehjem teflon tissue grinder (chamber clearance 0.1–0.15 mm) at top speed with five passes up and down hard. Subsequent centrifugation steps were performed exactly as described by Walter and Blobel (1983) with the exception that the rough microsome pellet was resuspended by pipetting up and down in a P1000 pipette tip (Gilson) in 1 ml of 50 mM HEPES, 250 mM sucrose, 1 mM dithiothreitol (pH 7.3) (buffer B). Crude rough microsomes were snap frozen in liquid nitrogen in 50 µl aliquots and stored at 70°C. Microsomes were thawed and diluted in ice-cold buffer B before use. No microsomes were used after more than one freeze–thaw cycle.

Enzyme Assays

Microsomes were thawed and diluted 3-fold in buffer B, and 12.5 µl was assayed for glucose 6-phosphatase using mannose 6-phosphate as the substrate in the presence and absence of 0.1% Triton X-100 at 37°C (Aronson and Touster, 1974). Enzyme activity is expressed as absorbance units at 820 nm.

Peptides and Antibodies

The labeled Sendai virus nucleoprotein peptide (FAPGNYPAL 324332) (Schumacher et al., 1991) was synthesized as a 3,5-diiodo-Tyr (Peninsula) containing precursor, using conventional Tboc chemistry. The peptide was dissolved in water and reduced under ^3H gas at New England Nuclear Du Pont (Boston). Subsequently, the material was analyzed by thin layer chromatography on silica plates using the solvent system N-butanol:2; pyridin:0.75; H_2O:1; acetic acid:0.25. Thin layer chromatography plates were dried and exposed to Kodak X-AR5 films. Fully reduced FAPGNYPAL was identified by comigration with unlabeled FAPGNYPAL, which was visualized by ninhydrin staining. The corresponding region of the thin layer chromatography plate was excised and eluted with 1% trifluoroacetic acid (2 rounds of 20 min at room temperature). After freeze drying, the purity of the material was checked by high pressure liquid chromatography and found to be greater than 95%. The specific activity was determined to be 10 Ci/mmol. The peptide was stored at a concentration of 0.3375 mM in 0.1 × phosphate-buffered saline at 70°C and thawed and diluted in water before use. The unlabeled peptides used were synthesized by the DuPont RaMPS system (except the 23-mer actin peptide, which was synthesized by automated Fmoc chemistry), and all were purified by high pressure liquid chromatography. Before use, peptides were dissolved in water, and concentrations of solubilized peptide were determined by BCA assay (Pierce).

The Y-3 antibody is a mouse IgG2a monoclonal antibody with affinity for a lumenal epitope of H-2K^b molecules (Hammerling et al., 1982). The SF1-1.1.1 antibody is a mouse IgG2a monoclonal antibody specific for a lumenal epitope of H-2K^d molecules (Loken and Stall, 1982). Both antibodies were purified on protein G columns and stored at 1 mg/ml in water.

Assays for TAP1 Function

Each data point is derived from a 50 µl assay containing 1.8 glucose 6-phosphatase units of microsomes, ATP mix (50 µM ATP, 250 µM UTP, 2.5 mM creatine phosphate, 8 U of rabbit muscle creatine kinase [Sigma]), 50 mM HEPES (pH 7.3), 150 mM KOAc, 5 mM MgOAc, 250 mM sucrose, 1 mM dithiothreitol, and peptides diluted in water. ATPγS was from Boehringer-Mannheim. Assays were assembled on ice, mixed by gentle agitation, incubated in 0.5 ml plastic microfuge tubes,

returned to ice, and rapidly pipetted onto the surface of 3 ml, ice-cold cushions of 1 M KOAc, 500 mM sucrose, 50 mM HEPES (pH 7.0), 10 μM unlabeled FAPGNYPAL in thick-walled polypropylene centrifuge tubes (Beckman #349622). The reaction was pelleted through the cushion in a TL.100.3 fixed-angle rotor at 60,000 rpm for 15 min at 4°C in a Beckman TL.100 ultracentrifuge. The supernatant was aspirated, and the pellet was resuspended in 100 ml of lysis buffer (phosphate-buffered saline [pH 7.3], 1% NP-40, 0.5 mM phenylmethylsulfonyl fluoride, 0.027 U/ml aprotinin) by repeated pipetting. Unlabeled FAPGNYPAL was included to 10 mM if the lysate was to be immunoprecipitated. The pellets were solubilized for 30 min on ice, and then debris was pelleted at 15,000 × g for 10 min at 4°C. Supernatants were mixed with 5 ml of Ecoscint (National Diagnostics) and counted.

For immunoprecipitation, supernatants were mixed with 5 μg of pure antibody and left on ice for 1 hr. Then 100 μl of a slurry of Gamma G beads (Pharmacia) in lysis buffer was added and mixed end over end for a further hour at 4°C. The beads were then washed three times in 200 μl of lysis buffer, each time collecting the beads by centrifugation at 15,000 × g for 15 s. The beads were then mixed with 5 ml of Ecoscint and counted.

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References

Aronson, N. N., and Touster, O. (1974). Isolation of rat liver plasma membrane fragments in isotonic sucrose. Meth. Enzymol. *31*, 90–102.

Ashton-Rickardt, P. G., Van Kaer, L., Schumacher, T. N. M., Ploegh, H. L., and Tonegawa, S. (1993). Peptide contributes to the specificity of positive selection of CD8+ T cells in the thymus. Cell *73*, 1041–1049.

Attaya, M., Jameson, S., Martinez, C. K., Hermel, E., Aldrich, C., Forman, J., Fischer-Lindahl, K., Bevan, M. J., and Monaco, J. J. (1992). Ham-2 corrects the class I antigen-processing defect in RMA-S cells. Nature *355*, 647–649.

Bijlmakers, M. K. E., Neefjes, J. J., Wojcik-Jacobs, E. H. M., and Ploegh, H. L. (1993). The assembly of H-2K^b class I molecules translated in vitro requires oxidized glutathione and peptide. Eur. J. Immunol., in press.

Blight, M. A., and Holland, I. B. (1990). Structure and function of hemolysin B, P-glycoprotein, and other members of a novel family of membrane translocators. Mol. Microbiol. *4*, 873–880.

Brown, M., Driscoll, J., and Monaco, J. J. (1991). Structural and serological similarity of MHC-linked LMP and proteosome (multicatalytic proteinase) complexes. Nature *353*, 355–357.

DeMars, R., Chang, C. C., Shaw, S., Reitnauer, P. J., and Sondel, P. M. (1984). Homozygous deletions that simultaneously eliminate expression of class I and class II antigens of EBV-transformed B-lymphoblastoid cells. Hum. Immunol. *11*, 77–97.

Deres, K., Schumacher, T. N. M., Weismuller, K.-H., Stevanovic, S., Greiner, G., Jung, G., and Ploegh, H. (1992). Preferred size of peptides that bind to H-2K^b is sequence-dependent. Eur. J. Immunol. *22*, 1603–1608.

Deverson, E. V., Gow, I. R., Coadwell, W. J., Monaco, J. J., Butcher, G. W., and Howard, C. H. (1990). MHC class II region encoding proteins related to the multidrug resistance family of membrane transporters. Nature *348*, 738–741.

Falk, K., Rotzschke, O., and Rammensee, H.-G. (1990). Cellular peptide composition governed by major histocompatibility complex class I molecules. Nature *348*, 248–251.

Falk, K., Rotzschke, O., Stevanovic, S., Jung, G., and Rammensee, H.-G. (1991). Allele-specific motifs revealed by sequencing of self-peptides eluted from MHC molecules. Nature *351*, 290–296.

Glynne, R., Powis, S. H., Beck, S., Kelly, A., Kerr, L. A., and Trowsdale, J. (1991). A proteasome-related gene between the two ABC transporter loci in the class II region of the human MHC. Nature *353*, 357–360.

Gros, P., Croop, J., and Housman, D. (1986). Mammalian multidrug resistance gene: complete cDNA sequence indicates strong homology to bacterial transport proteins. Cell *47*, 371–380.

Hammerling, G. J., Rusch, E., Tada, N., Kimura, S., and Hammerling, U. (1982). Localization of allodeterminants on H-2K^b antigens determined with monoclonal antibodies and H-2 mutant mice. Proc. Natl. Acad. Sci. USA *79*, 4737–4741.

Hiles, I. D., Gallagher, M. P., Jamieson, D. J., and Higgins, C. F. (1987). Molecular characterization of the oligopeptide permease of *Salmonella typhimurium*. J. Mol. Biol. *195*, 125–142.

Hyde, S. C., Emsley, P., Hartshorn, M. J., Mimmack, M. M., Gileadi, U., Pearce, S. R., Gallagher, M. P., Gill, D. R., Hubbard, R. E., and Higgins, C. F. (1990). Structural model of ATP-binding proteins associated with cystic fibrosis, multidrug resistance and bacterial transport. Nature *346*, 362–365.

Kelly, A., Powis, S. H., Glynne, R., Radley, E., Beck, S., and Trowsdale, J. (1991). Second proteasome related gene in the human MHC class II region. Nature *353*, 667–668.

Kelly, A., Powis, S., Kerr, L.-A., Mockridge, I., Elliott, T., Bastin, J., Uchanska-Ziegler, B., Ziegler, A., Trowsdale, J., and Townsend, A. (1992). Assembly and function of the two ABC transporter proteins encoded in the human major histocompatibility complex. Nature *355*, 641–644.

Kleijmeer, M. J., Kelly, A., Geuze, H. J., Slot, J. W., Townsend, A. R., and Trowsdale, J. (1992). Location of MHC-encoded transporters in the endoplasmic reticulum and cis-Golgi. Nature *357*, 342–344.

Koppelman, B., Zimmerman, D. L., Walter, P., and Brodsky, F. M. (1992). Evidence for peptide transport across microsomal membranes. Proc. Natl. Acad. Sci. USA *89*, 3908–3912.

Lévy, F., Larsson, R., and Kvist, S. (1991a). Translocation of peptides through microsomal membranes is a rapid process and promotes assembly of HLA-B27 heavy chain and β2 microglobulin translated in vitro. J. Cell Biol. *115*, 959–970.

Lévy, F., Gabathuler, R., Larsson, R., and Kvist, S. (1991b). ATP is required for in vitro assembly of MHC class I antigens but not for transfer of peptides across the ER membrane. Cell *67*, 265–274.

Ljunggren, H.-G., and Karre, K. (1985). Host resistance directed selectively against H-2 deficient lymphoma variants: analysis of the mechanism. J. Exp. Med. *162*, 1745–1759.

Ljunggren, H.-G., Stam, N. S., Ohlen, C., Neefjes, J. J., Hogland, P., Heemels, M. T., Bastin, J., Schumacher, T. N. M., Townsend, A., Karre, K., and Ploegh, H. L. (1990). Empty class I molecules come out in the cold. Nature *346*, 476–480.

Lobigs, M., and Mullbacher, A. (1993). Recognition of vaccinia virus-encoded major histocompatibility complex class I antigens by virus immune cytotoxic T cells is independent of the polymorphism of the peptide transporters. Proc. Natl. Acad. Sci. USA *90*, 2676–2680.

Loken, M. R., and Stall, A. M. (1982). Flow cytometry as an analytical and preparative tool in immunology. J. Immunol. Meth. *50*, R85–R112.

Martinez, C. K., and Monaco, J. J. (1991). Homology of proteasome subunits to a major histocompatibility complex-linked LMP gene. Nature *353*, 664–667.

McGrath, J. P., and Varshavsky, A. (1989). The yeast *STE6* gene encodes a homologue of the mammalian multidrug resistance p-glycoprotein. Nature *340*, 400–404.

Momburg, F., Ortiz-Navarette, V., Neefjes, J., Goulmy, E., van der Wal, Y., Spits, H., Powis, S. J., Butcher, G. W., Howard, J. C., Walden, P., and Hammerling, G. (1992). The proteasome subunits encoded by the major histocompatibility complex are not essential for antigen presentation. Nature *360*, 174–177.

Monaco, J. J., Cho, S., and Attaya, M. (1990). Transport protein genes

in the murine MHC: possible implications for antigen processing. Science *250*, 1723–1726.

Neefjes, J. J., Schumacher, T. N. M., and Ploegh, H. L. (1991). Assembly and intracellular transport of major histocompatibility complex molecules. Curr. Opin. Cell Biol. *3*, 601–609.

Ortiz-Navarette, V., and Hammerling, G. J. (1991). Surface appearance and instability of empty H2 class I molecules under physiological conditions. Proc. Natl. Acad. Sci. USA *88*, 3594–3597.

Powis, S. J., Townsend, A. R. M., Deverson, E. V., Bastin, J., Butcher, G. W., and Howard, J. C. (1991). Restoration of antigen presentation to the mutant cell line RMA-S by an MHC-linked transporter. Nature *354*, 528–531.

Powis, S. J., Deverson, E. V., Coadwell, W. J., Ciruela, A., Huskisson, N. S., Smith, H., Butcher, G. W., and Howard, J. C. (1992). Effect of polymorphism of an MHC-linked transporter on the peptides assembled in a class I molecule. Nature *357*, 211–215.

Riordan, J. R., Rommens, J. M., Kerem, B.-T., Alon, N., Rozmahel, R., Grzelczak, Z., Zielenski, J., Lok, S., Plavsic, N., Chou, J.-L., Drumm, M. L., Iannuzzi, M. C., Collins, F. S., and Tsui, L.-C. (1989). Identification of the cystic fibrosis gene: cloning and characterization of complementary DNA. Science *245*, 1066–1073.

Rotzschke, O., Falk, K., Deres, K., Schild, H., Norda, M., Metzger, J., Jung, G., and Rammensee, H.-G. (1990). Isolation and analysis of naturally processed viral peptides as recognized by cytotoxic T cells. Nature *348*, 252–254.

Schumacher, T. N. M., Heemels, M.-T., Neefjes, J. J., Kast, W. M., Melief, C. J. M., and Ploegh, H. L. (1990). Direct binding of peptide to empty MHC class I molecules on intact cells and in vitro. Cell *62*, 563–567.

Schumacher, T. N. M., de Bruijn, M. L. H., Vernie, L. N., Kast, W. M., Melief, C. J. M., Neefjes, J. J., and Ploegh, H. L. (1991). Peptide selection by MHC class I molecules. Nature *350*, 703–706.

Spies, T., and DeMars, R. (1991). Restored expression of major histocompatibility class I molecules by gene transfer of a putative peptide transporter. Nature *351*, 323–324.

Spies, T., Bresnahan, M., Bahram, S., Arnold, D., Blanck, G., Mellins, E., Pious, D., and DeMars, R. (1990). A gene in the major histocompatibility complex class II region controlling the class I antigen presentation pathway. Nature *348*, 744–747.

Spies, T., Cerundolo, V., Colonna, M., Cresswell, P., Townsend, A., and DeMars, R. (1992). Presentation of viral antigen by MHC class I molecules is dependent on a putative peptide transporter heterodimer. Nature *355*, 644–646.

Townsend, A., Ohlen, C., Bastin, J., Ljunggren, H.-G., Foster, L., and Karre, K. (1989). Association of class I major histocompatibility heavy and light chains induced by viral peptides. Nature *340*, 443–448.

Townsend, A., Elliott, T., Cerundolo, V., Foster, L., Barber, B., and Tse, A. (1990). Assembly of MHC class I molecules analyzed in vitro. Cell *62*, 285–295.

Trowsdale, J., Hanson, I., Mockridge, I., Beck, S., Townsend, A., and Kelly, A. (1990). Sequences encoded in the class II region of the MHC related to the 'ABC' superfamily of transporters. Nature *348*, 741–744.

Udaka, K., Tsomides, T. J., and Eisen, H. N. (1992). A naturally occurring peptide recognized by alloreactive $CD8^+$ cytotoxic T lymphocytes in association with a class I MHC protein. Cell *69*, 989–998.

Van Kaer, L., Ashton-Rickardt, P. G., Ploegh, H. L., and Tonegawa, S. (1992). *TAP1* mutant mice are deficient in antigen presentation, surface class I molecules, and $CD4^-8^+$ T cells. Cell *71*, 1205–1214.

Walter, P., and Blobel, G. (1983). Preparation of microsomal membranes for cotranslational protein translocation. Meth. Enzymol. *96*, 84–93.

Yewdell, J., Esquivel, F., Arnold, D., Spies, T., Eisenlohr, L. C., and Bennink, J. R. (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*, 1785–1790.

Zweerink, H. J., Gammon, M. C., Utz, U., Sauma, S. Y., Harrer, T., Hawkins, J. C., Johnson, R. P., Sirotina, A., Hermes, J. D., Walker, B. D., and Biddison, W. E. (1993). Presentation of endogenous peptides to MHC class I-restricted cytotoxic T lymphocytes in transport deletion mutant T2 cells. J. Immunol. *150*, 1763–1771.