8. Each strand of pbEG4 was sequenced twice with both normal and a mixture of deaza–deoxyadenosine triphosphate and deaza–deoxyguanosine triphosphate, and specific oligonucleotide primers. The cDNA of pbEG4 is 1008 nucleotides long, and its sequence has been submitted to EMBL, GenBank, and DDBJ nucleotide sequence databases under the accession number X72802.
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13. The immunizing synthetic peptide had the additional KC sequence at the COOH-terminus for solubility and cross-linking purposes. Preimmune and immune rabbit immunoglobulin G (IgG) were purified by ammonium sulfate precipitation and CM Affi-gel Blue (Bio-Rad) chromatography. For purification of the antibody by affinity chromatography, the peptide was cross-linked to bovine serum albumin and coupled to cyanogen bromide–activated Sepharose (Pharmacia). Antibodies bound to the resin were eluted with 3.5 M $MgCl_2$, 10 mM sodium phosphate (pH 7.3).
14. The expression vector pSVT7EG was constructed by ligation of the Eco RI fragment of pbEG4 at the Eco RI site of pSVT7 [P. Bird, M.-J. Gething, J. Sambrook, *J. Cell Biol.* **105**, 2905 (1987)]. The two orientations of the cDNA were obtained. For pSVT7tEG, the sequences of the cDNA coding for amino acids 1 through 48 were deleted by site-directed mutagenesis using PCR. The mutagen sense primer was TCTGAATTCGCCATGGCGG-GGCTTCCCGCGGTGCCA, where the underlined nucleotides surrounding the initiator codon conformed to Kozak's rule [M. Kozak, *Cell* **44**, 283 (1986)]. The antisense primer was ATCGAATTC-GTCCGCCCCAAGGACTTTTCAG. Vent polymerase (NEB) was used for amplification according to the manufacturer's instructions. Thirty cycles of amplification were used, each cycle being 94°C for 1 min, 72°C for 2 min. Amplified DNA was digested with Eco RI and ligated to the Eco RI site of pSVT7. The constructs were verified by DNA sequencing.
15. Exponentially growing COS-7 cells (5×10^5 per 78 cm^2 dish) were transfected for 20 hours with 25 μg of the Endo G expression vectors and 4.5 μg of pSV_2CAT by the calcium phosphate method. Cells were harvested 48 hours after transfection and extracted (*1*). Chloramphenicol acetyltransferase (CAT) activity was used to normalize the efficiency of transfection.
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19. Cells on cover slides were fixed with 3.7% formaldehyde in phosphate-buffered saline and post-fixed with methanol and 90% acetone. Cells were reacted with affinity-purified rabbit antibodies to Endo G and MAB1273 (Chemicon, Inc.) Fluorescein isothiocyanate (FITC)–labeled goat antibody and Texas red–labeled donkey antibody were used as secondary antibodies. Calf liver samples were obtained from a local slaughterhouse, frozen within 10 min of the animal's death, and sliced into sections of about 10 μm in thickness. Slides were observed with a Bio-Rad MRC-600 confocal imaging system mounted on a Nikon Diaphot-TMD (×60 objective lens with a 1.4 numerical aperture). A 630-nm filter was used for dual labeling to cut FITC emission in the Texas red range.
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39. We thank C. Lazure and M. Blum for NH_2-terminal sequence determination, H. P. Zassenhaus for *S. cerevisiae* mitochondria from wild-type and *Nuc1* null mutants, M. Fraser for *N. crassa* endo-exonuclease and for rabbit antisera against it, D. A. Clayton for plasmid pMR718B, S. St.-Pierre for oligopeptide synthesis, T. Moss for revision of the manuscript, S. Rioux for pBT29, S. Descôteaux for DNA sequencing, E. Belzile for cell fractionation and marker determinations, C. Chamberland for confocal microscopy, M. Lambert for oligonucleotide synthesis, and G. Langlois and P. Paquin for photographic reproduction. Supported by the Medical Research Council of Canada (doctoral fellowship to J.C. and scholarship to A.R.-C.) and by a grant from the National Cancer Institute of Canada.

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Selective and ATP-Dependent Translocation of Peptides by the MHC-Encoded Transporter

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Major histocompatibility complex (MHC) class I molecules present peptides derived from nuclear and cytosolic proteins to $CD8^+$ T cells. These peptides are translocated into the lumen of the endoplasmic reticulum (ER) to associate with class I molecules. Two MHC-encoded putative transporter proteins, TAP1 and TAP2, are required for efficient assembly of class I molecules and presentation of endogenous peptides. Expression of TAP1 and TAP2 in a mutant cell line resulted in the delivery of an 11–amino acid oligomer model peptide to the ER. Peptide translocation depended on the sequence of the peptide, was adenosine triphosphate (ATP)–dependent, required ATP hydrolysis, and was inhibited in a concentration-dependent manner.

Cytotoxic T lymphocytes recognize peptides presented by MHC class I molecules. It is likely that two steps precede the binding by class I molecules of peptides originating from cytosolic or nuclear antigens. First, proteins undergo a limited degradation in which the multicatalytic or proteasome complex may be involved (*1*). Second, the resulting peptides are translocated from the cytosol into the lumen of the ER. Analysis of mutant cell lines with defective class I assembly and antigen presentation led to the identification of two related genes, located in the MHC class II region of humans and rodents, that encode the transporters associated with antigen processing, TAP1 and TAP2. These proteins form heterodimers (*2*, *3*) and are expressed in the ER membrane (*4*). By structural homology they belong to a family of transporter proteins that have multiple membrane-spanning sequences and contain an ATP-binding consensus sequence (*5*). Other members are the mammalian P-glycoproteins (multidrug resistance pumps), the cystic fibrosis transmembrane conductance regulator (CFTR), bacterial hemolysin transporters (HLyB), and the yeast protein Ste6.

Two lines of evidence suggested that the TAP1-TAP2 heterodimer translocates peptides to the ER: The reconstitution of class I assembly (*2*, *3*, *6*–*9*) in mutant cells transfected with TAP genes and the observation that expression of different alleles of

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rat TAP2 results in a different spectrum of peptides associated with the RT1A^a MHC class I molecule (*10*). However, no direct evidence has been provided so far. Indeed, the function of TAP1 and TAP2 as ATP-dependent peptide transporters has been questioned. ATP-independent peptide translocation was observed in microsomes isolated from both the cell line T2, which has a deletion encompassing TAP1 and TAP2, and the parental cell line T1 (*11*). ATP-independent peptide translocation was also observed in experiments using microsomes isolated from dog pancreas (*12*). In both these studies it was assumed that microsomes are not leaky for small molecules like peptides.

We assessed peptide translocation in the TAP-deficient cell line T2 and in T2 transfected with rat TAP1 and TAP2 (T2.TAP1+2). We have previously shown stable assembly of class I molecules and presentation of endogenously derived peptides in T2.TAP1+2 cells (9). To obtain access to the cytosol without penetrating the ER membrane, we permeabilized the cell surface with the bacterial toxin streptolysin O. The cells were then incubated with the model peptide GP (sequence, RYWANATRSGG) (*13*) that was labeled by iodination on tyrosine (Y). This 11–amino acid–containing oligomer (11-mer) peptide contains an N-linked glycosylation site (NAT). The addition of the N-linked glycan takes place in the ER (*14*). N-linked glycosylation of the GP peptide was therefore used to monitor translocation to the ER (*15*). Sepharose beads conjugated with the lectin concanavalin A (Con A) were used to specifically recover the glycosylated peptide. Any difference between T2 and T2.TAP1+2 in the extent of glycosylation of the model peptide can be attributed only to translocation of this peptide to the ER by TAP1 and TAP2.

T2 and T2.TAP1+2 were permeabilized by streptolysin O and incubated with ^{125}I-GP for 10 min at 37°C in the absence or presence of different concentrations of ATP or of the nonhydrolyzable ATP analogs ATP-γ-S, AMP-PCP, and AMP-PNP (*15*). The cells were lysed with NP-40. Glycosylated peptides were recovered by Con A–Sepharose binding and eluted with α-methylmannoside before quantitation in a gamma counter (Fig. 1). Glycosylated ^{125}I-GP could be recovered from T2.TAP1+2 cells but not from T2 cells. Translocation to the ER followed by glycosylation of ^{125}I-GP used ATP in a concentration-dependent manner. Nonhydrolyzable ATP analogs did not yield glycosylated ^{125}I-GP, suggesting that hydrolysis of ATP is required for TAP1+2-dependent peptide translocation to the ER. In T2 cells transfected with either TAP1 or TAP2 alone (9) no peptide translocation was observed (*16*).

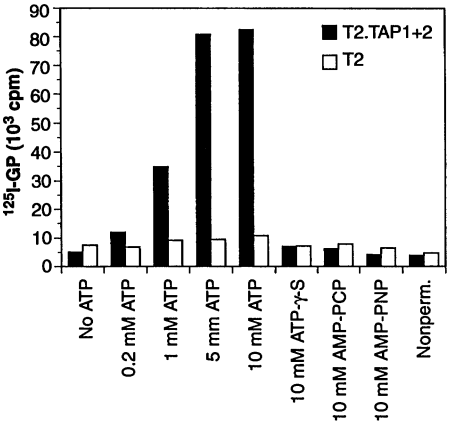

Fig. 1. ATP-dependent peptide translocation in T2.TAP1+2 transfectants. The plasma membrane of 3.5 × 10^6 T2 or T2.TAP1+2 cells was permeabilized with streptolysin O, and cells were incubated for 10 min at 37°C with ~100 ng of ^{125}I-GP in the absence or presence of different concentrations of ATP or nonhydrolyzable ATP analogs, as indicated. Right bar, nonpermeabilized cells. The peptides that are translocated into the ER become glycosylated. These were isolated by Con A–Sepharose, eluted from Con A–Sepharose by α-methylmannoside, and quantitated by gamma counting, as described (*15*). Translocation of peptides to the ER is ATP-dependent and occurs only in cells expressing the peptide transporter subunits.

The rate of translocation of ^{125}I-GP in permeabilized T2.TAP1+2 was determined at 37°C in the presence or absence of saturating amounts of ATP (10 mM, Fig. 1). The cells were lysed and glycosylated ^{125}I-GP recovered (Fig. 2). Rapid translocation of ^{125}I-GP to the ER was observed during the first 10 min and then leveled off. Addition of ATP after 10 min had no significant effect on the recovery of glycosylated ^{125}I-GP, whereas a second addition of ^{125}I-GP after 10 min led to an increased recovery of glycosylated peptide (*16*). This indicates that the observed plateau in Fig. 2 is not due to lack of ATP or the lipid-linked oligosaccharide donor in the ER but probably due to degradation of the input peptide in the cytosol.

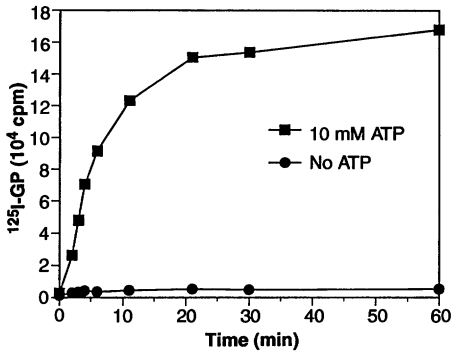

Fig. 2. Kinetics of peptide translocation in T2.TAP1+2 transfectants. T2.TAP1+2 cells (5 × 10^6 per sample) were permeabilized with streptolysin O. ^{125}I-GP (~100 ng) was added, and the cells were incubated at 37°C in the absence or presence of 10 mM ATP for 0, 2, 3, 4, 6, 11, 21, 30, or 60 min. Cells were lysed, and the glycosylated peptides were isolated and quantitated (*15*). Translocation of ^{125}I-GP is rapid during the first 10 min and then levels off.

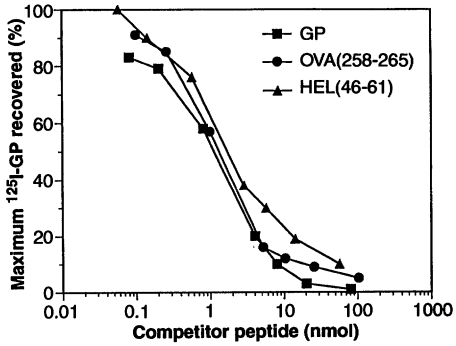

Fig. 3. Competition of peptide translocation with other peptides. T2.TAP1+2 cells (5 × 10^6 per sample) were permeabilized with streptolysin O. ^{125}I-GP (100 ng) was mixed with different amounts of unlabeled peptides GP, OVA(258-265), or HEL(46-61) (*19*). The permeabilized T2.TAP1+2 cells were incubated with the respective peptide mixtures at 37°C for 10 min in the presence of 10 mM ATP. The cells were lysed and glycosylated ^{125}I-GP was recovered (*15*). Fifty percent inhibition is obtained with 1 nmol of OVA(258-265) or GP peptide and with 2 nmol of HEL(46-61).

A peptide supply system for MHC class I molecules will have to translocate many different peptides. We therefore competed for translocation of ^{125}I-GP with different concentrations of GP (the input 11-mer

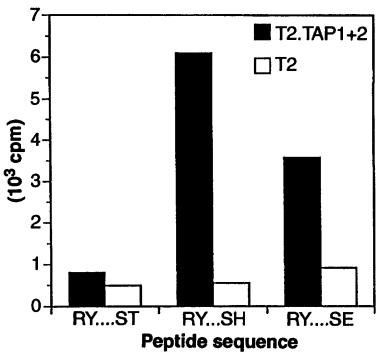

Fig. 4. Selection of peptides by the peptide transporter. T2 or T2.TAP1+2 cells (2 × 10^6 per sample) were permeabilized with streptolysin O and incubated for 20 min in the presence of 10 mM ATP and ~300 ng of either iodinated RYWANATRSE (RY . . . SE), RYWANATRSH (RY . . . SH), or RYWANATRST (RY . . . ST) (*17*). The glycosylated peptides were recovered as described and quantitated (*15*). Peptides with H or E as the COOH-terminal residue are efficiently translocated; the peptide with T as the COOH-terminal residue is not.

peptide), the 8-mer peptide ovalbumin (258-265) [OVA(258-265)], and the 16-mer peptide hen egg lysozyme (46-61) [HEL(46-61)] in the presence of 10 mM ATP at 37°C. GP and OVA(258-265) competed with equal efficiency for translocation of ^{125}I-GP (50% inhibition at ~1 nmol competitor), and HEL(46-61) required slightly higher concentrations (50% inhibition at ~2 nmol competitor) (Fig. 3). It is not clear whether these differences are due to the length or sequence of the competing HEL peptide.

We assessed the specificity of the peptide transporter for different peptides using the translocation of the 10-mer peptide RYWANATRSX, where X is either T, H, or E (*13*). Similar amounts of iodinated peptide (*17*) were added to permeabilized T2 or T2.TAP1+2 cells, and translocated peptides were recovered (Fig. 4). The peptides with the COOH-terminal residue H or E were efficiently translocated by T2.TAP1+2, but the peptide with T as the COOH-terminal residue was not. This suggests that the peptide transporter selects peptides, in agreement with Powis *et al.* (*10*), and indicates that the COOH-terminal residue is essential for this selection.

From the competition experiment with T2.TAP1+2 cells (Fig. 3), we estimate a minimal translocation rate of ~2×10^4 GP molecules per minute (*18*). It is uncertain whether the rate of peptide transport is exclusively controlled by the peptide transporters or whether additional cytosolic molecules, such as chaperonins, are involved. The amount of TAP1 and TAP2 expressed in transfected T2 cells is similar to that of stimulated rat spleen cells (*9*), and therefore the calculation can be considered as representative of physiological cells. Although it has not been determined whether the concentrations of input peptide [up to 2 μM for maximal translocation (Fig. 3)] used in our study can be reached in a living cell, the calculation indicates that peptide transporters allow translocation of large amounts of peptides to the ER. MHC class I molecules bind only a selected set of peptides with high affinity, so transport of large quantities of peptides may be essential. In addition, efficient and rapid translocation of peptides to the ER may be required to escape complete breakdown in the cytosol. The system described here should allow the elucidation of the length and sequence constraints for peptide transport as well as the possible degradation of peptides in the ER.

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13. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; N, Asn; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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15. T2 cells and T2 cells transfected with rat TAP1 and TAP2 (T2.TAP1+2) (*9*) were cultured in RPMI supplemented with 10% fetal calf serum. The cells were permeabilized with streptolysin O (2 IU/ml) in 100 μl of incubation buffer [130 mM KCl, 10 mM NaCl, 1 mM $CaCl_2$, 2 mM EGTA, 2 mM $MgCl_2$, 5 mM Hepes (pH 7.3)]. Routinely, 60 to 90% of the cells were permeabilized as determined by trypan blue uptake. As a control, nonpermeabilized cells were incubated in the same buffer. GP peptide (10 μg) was iodinated by chloramine T with 1 mCi of Na^{125}I. Free iodine was separated from bound iodine by DOWEX (OH^-). The cells in 100 μl of permeabilization buffer were incubated for 10 min at 37°C with 10 μl (~100 ng = 0.075 nmol) of ^{125}I-GP in the presence or absence of (freshly prepared) ATP or 10 mM adenosine 5′-*O*-(3-thiotriphosphate) lithium salt (ATP-γ-S), 5′-adenylylimidodiphosphate (AMP-PNP), or β,γ-methyleneadenosine 5′-triphosphate lithium salt (AMP-PCP) (all from Sigma). The reaction was terminated by addition of 1 ml NP-40 lysis mix [1% NP-40, 150 mM NaCl, 5 mM $MgCl_2$, and 50 mM tris-HCl (pH 7.5)], nuclei were removed, and the lysate was incubated with 100 μl of packed Con A–Sepharose for 1 hour. The Con A–Sepharose was washed five times with NP-40 lysis mix, and the bound peptides were eluted with 100 mM α-methylmannoside. The eluted fractions were quantitated by gamma counting.
16. J. J. Neefjes, F. Momburg, G. J. Hämmerling, unpublished data.
17. Peptides were synthesized with a free COOH-terminus. They were iodinated by chloramine T–catalyzed iodination with 500 μCi of Na^{125}I. Specific activity was as follows: RY . . . ST, 130 μCi/10 μg; RY . . . SE, 153 μCi/10 μg; and RY . . . SH, 143 μCi/10 μg.
18. In all experiments using 5×10^6 T2.TAP1+2 cells in a 100-μl reaction volume, about 1% of the iodinated input peptide could be recovered from Con A–Sepharose. Because no competition for translocation of ^{125}I-GP is observed when 0.2 nmol of competitor peptide is added (Fig. 3), it can be estimated that 5×10^6 cells can translocate 1% of input GP, corresponding to 2 pmol of GP per 10 min, or 2×10^4 peptides per T2.TAP1+2 cell per min. This calculation is a minimal estimate, and it is possible that the actual rate of translocation is higher.
19. The sequence of OVA(258-265) is SIINFEKL, and the sequence of HEL(46-61) is NTDGSTDYGILQINSR (*13*). These peptides do not contain an N-linked glycosylation site. OVA(258-265) can bind to the H-2K^b MHC class I molecule, and HEL(46-61) to the A^k class II molecule.

20. We thank M. J. Bijlmakers and H. L. Ploegh for samples of GP peptide, J. Roelse for experimental support, and the laboratory of J. Howard and G. Butcher for providing rat TAP1 and TAP2^a cDNAs. Supported by an EMBO long-term fellowship (to J.J.N.) and by an EMBO short-term fellowship (to F.M.).

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