Peptide Size Selection by the Major Histocompatibility Complex-encoded Peptide Transporter

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Summary

The major histocompatibility complex (MHC)–encoded heterodimeric TAP1/TAP2 transporter (TAP) translocates cytosolic peptides into the lumen of the endoplasmic reticulum (ER), where peptides of 8 to 11 amino acids long associate with MHC class I molecules. We have studied the selectivity of peptide translocation by TAP in streptolysin O–permeabilized cells using glycosylatable, radioiodinated model peptides to detect import into the ER lumen. TAP-dependent translocation of a radiolabeled nonamer peptide was most efficiently inhibited by unlabeled 9- to 11-mer peptides. Peptides between 7 and 40 amino acids long all could inhibit transport, the longer peptides being least effective. Also, peptides shorter than eight amino acids were inefficiently translocated. The use of directly labeled length variants in translocation assays and TLC analysis of the transported material revealed two pathways for translocation: short peptides (7 to 13 amino acids long) were translocated without prior modification. In contrast, transport of longer peptides was not effective. Instead such peptides were clipped by cytosolic peptidases before efficient transport. Our data suggest that TAP preferentially translocates peptides of appropriate length for class I binding. Furthermore, TAP-translocated peptides were rapidly released from the ER unless they were trapped there by being glycosylated or by binding to MHC class I molecules.

M HC class I molecules present peptides derived from nuclear or cytosolic proteins to CD8^{+} cytotoxic T cells. Naturally processed class I–bound peptides usually have a length of 8–11 amino acids (1) and are an integral part of the structure of MHC class I molecules (2, 3). Class I molecules associate with peptide in the lumen of the endoplasmic reticulum (ER)[1] (4–6). Because most of these peptides are generated from cytosolic or nuclear proteins, this implies that the peptides have to be translocated over the ER membrane. The characterization of mutant cell lines expressing "unstable" class I molecules unless loaded with exogenous peptides led to the characterization of two genes that are required for proper peptide loading of class I molecules (7–13). The gene products were named TAP1 and TAP2 for Transporters Associated with Antigen Presentation. Both TAP1 and TAP2 are multimembrane spanning proteins with an ATP-binding cassette and are associated with one another in the ER membrane (14, 15). We recently showed that TAP1/TAP2 complexes

(TAP) translocates peptides into the ER and that this process requires the hydrolysis of ATP (16).

Proteolytic processing of cytosolic and nuclear proteins precedes the translocation of peptides by TAP. This process is still ill defined. The cytosolic multicatalytic proteinases (proteasome) may be involved in this degradation process (17), but the evidence is still circumstantial. Introduction of antigen into the ubiquitin-dependent degradation system (the 26S form of the proteasome) increased the efficiency of antigen presentation (18). Temperature-sensitive mutant cells defective in ubiquitin-dependent proteolysis only presented antigen efficiently when cultured at the permissive temperature (19). Interestingly, the TAP genes are closely located to two genes encoding the Lmp2 and Lmp7 subunits of the multicatalytic proteasome (20–23). Although this initially suggested the involvement of the proteasome in the generation of presentable peptides, transfection studies have shown that these proteasome subunits are not essential for antigen presentation (24, 25). Involvement of other cytosolic or membrane-bound proteases in antigen presentation has not been shown, but neither has it been excluded. The processes that follow the generation of antigenic fragments in the cytosol are unclear. TAP translocates peptides into the lumen of the

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[1] Abbreviations used in this paper: Endo H, endoglycosidase H; ER, endoplasmic reticulum; glc, glucose; HK, hexokinase; TAP, transporter associated with antigen presentation.

ER (16, 26, 27) but whether there is any selection for size is not known. Furthermore, it is unclear what the fate of the peptides in the ER lumen is.

We have reconstituted TAP-dependent translocation of peptides into the lumen of the ER in streptolysin O-permeabilized cells (16). Here we show that model peptides of 9–11 amino acids long are transported most efficiently and that the affinity of longer peptides decreases with increasing size. We found longer peptides (16 or more amino acids) not to be transported efficiently unless first truncated to shorter peptides. Also, peptides that were somewhat longer (12–13 amino acids) or shorter (7 amino acids) than the naturally class I–associated peptides were translocated. Once the peptides have arrived in the ER lumen, they are rapidly released (unless they associate with ER accessory molecules or class I molecules). Thus, TAP selects for peptide lengths that cover the size of peptides found associated with class I molecules. Because peptides are only transiently present in the ER lumen, class I molecules have to bind these peptides with high affinity.

Materials and Methods

Cell Lines. The following cell lines were used: the TAP-deficient mutant TxB cell hybrid T2 (28); the parental human B-lymphoblastoid cell line LCL 721, and T2 cells transfected with rat TAP1 and TAP2[a] (T2/TAP1+2; see reference 24). Cells were maintained in RPMI 1640 supplemented with 10% FCS.

Peptides. The following peptides were used:

	Code	Sequence
1.	#49	NATY
2.	#50	ANATY
3.	#53	RYWANATY
4.	#54	TRYWANATY
5.	#55	RYWANATRSA
6.	#72	RYWANATRSY
7.	#78	KTWANATRSYE
8.	#146	VDNKTAY
9.	#147	TVDNKTRAY
10.	#148	ETVDNKTFRAY
11.	#149	IETVDNKTSFRAY
12.	#150	QIETVDNKTPSFRAY
13.	#88	[GS]$_2$SRYWANATRSGG
14.	#89	[GS]$_4$SRYWANATRSGG
15.	#90	[GS]$_{14}$SRYWANATRSGG
16.	SN11	RYWANATRSGG
17.	SN12A1	AYWANATRSG
18.	SN12$^-$	YWANATRSG

Peptides 1–14 were synthesized by f-moc peptide chemistry using a AMS 422 Multiple Peptide Synthesizer (ABIMED, Langenfeld, Germany) and peptides 15 and 16 were synthesized by t-boc chemistry on a peptide synthesizer (SAM2; Biosearch, a division of Millipore Corp., Bedford, MA). Peptides 1–4 and 13–16 were synthesized with an amidated COOH terminus. The peptides were analyzed by HPLC and TLC and were >95% pure.

10 μg peptide was radiolabeled at unique Tyr residues using chloramine T-catalyzed iodination (29) and free iodine was removed by anion exchange chromatography (DOWEX[OH-]). The specific activity of the iodinated peptides ranged from 20–50 μCi/μg. The iodinated peptides were stored at −20°C.

Peptide Translocation. Peptide translocation was performed as described (16). Briefly, 2.5–5 × 10^6 cells/incubation were harvested and washed once with 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). Cells were permeabilized with 2 IU streptolysin O/ml (Wellcome Reagent Ltd., Beckenham, UK) in 50 μl incubation buffer for 10 min at 37°C followed by the addition of 10 μl 100 mM ATP (Boehringer), 10–20 μl radioiodinated peptide and additional incubation buffer to a final volume of 100 μl. Peptide translocation was inhibited by lysing the permeabilized cells with 1 ml NP-40 lysis mix. Nuclei were removed and the glycosylated peptides were recovered with Con A–Sepharose (Pharmacia, Uppsala, Sweden), as described (16).

In competition experiments 2.5 × 10^6 T2/TAP-1+2 cells/sample were permeabilized for 10 min at 37°C with streptolysin O, as described (16). Cytosol and nonbound streptolysin O were removed by washing the cells three times with PBS supplemented with 100 μM glucose, 1 mM MgCl$_2$, 1 mM CaCl$_2$, and 1 mg/ml BSA. Cells were then distributed over tubes containing 100 ng radioiodinated peptide #147 (~2 μCi), the different concentrations of competitor and 10 mM ATP (pH 7). The final volume was 100 μl. The samples were incubated for 5 min at 37°C, followed by lysis, recovery of the glycosylated radiolabeled peptide #147 by Con A–Sepharose and quantitation by gamma counting.

The degradation of input peptides was followed by injecting 2-μl samples of the above described reaction mixture (i.e., permeabilized cells, ATP, and input peptide) at different times into 20 μl TLC buffer, 10-μl samples were analyzed by TLC. ATP depletion was used to terminate peptide translocation. This was achieved by adding 0.2 μl 1,500 U/ml hexokinase (Boehringer) and 10 μl 2 M glucose to the reaction mixture. The fate of nonglycosylated peptides in the ER was followed after inhibition of N-linked glycosylation by culturing the cells in the presence of 10 μg/ml tunicamycin (Sigma Chemical Co., St. Louis, MO) for 45 min before permeabilization with streptolysin O. The permeabilized cells were incubated with peptide #88 in the presence of 10 mM ATP for different periods, followed by five washes with PBS at 0°C. The cells were then extracted with 100 μl phenol and the peptides retained in the phenol phase were quantitated by gamma counting. 20 μl of the phenol phase (that contained most peptides) was analyzed by TLC. TLC plates were exposed to Kodak XAR 5 film.

Gel Electrophoresis and Peptide Analysis. Tricine SDS-PAGE was performed as described (30). After electrophoresis, the gel was immediately frozen and exposed to Kodak XAR-5 film. TLC was performed on silica-gel plates (Kieselgel 60; Merck Sharpe & Dohme, St. Louis, MO) in n-butanol/pyridine/acetic acid/water (97:75:15:60:) followed by autoradiography. Glycosylated peptides were analyzed on TLC after removal of the N-linked glycan (except for the Asn-linked GlcNAc) with endoglycosidase H (Endo H). The glycosylated peptides were first released from the Con A–Sepharose by boiling for 10 min in 0.1% SDS. Released peptides were then incubated overnight at 37°C in 50 μl 20 mM Na-citrate buffer, pH 5.2, including 2 mU Endo H (Boehringer GmbH, Mannheim, Germany).

The size of the translocated peptides was determined by Edman degradation using a sequencer (model A75A; Applied Biosystems, Foster City, CA), followed by gamma counting of the amino acids released after every Edman degradation step.

Results

Translocation of Peptides of Different Lengths by TAP. Translocation of peptides from the cytosol to the lumen of the ER by TAP was studied as described (16). Iodinated peptides
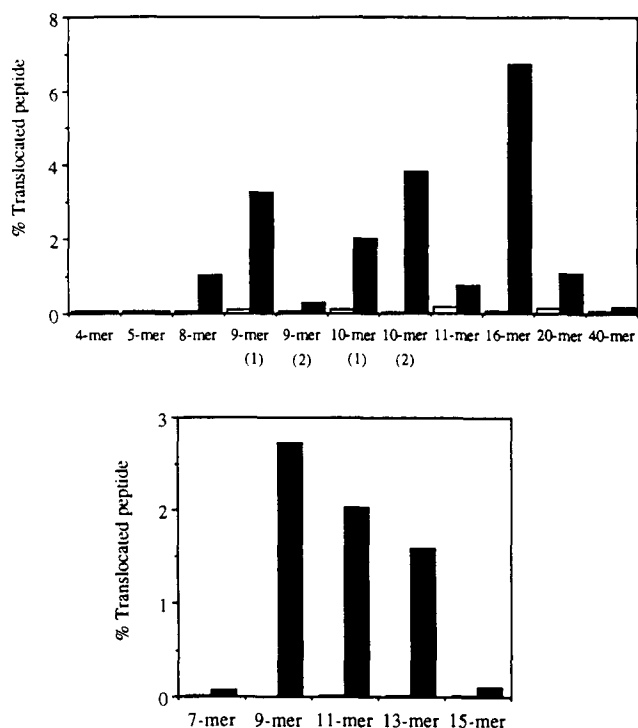
Figure 1. TAP-dependent peptide translocation of input peptides of different length. Peptides of different size were translocated for 20 min in the presence of ATP by streptolysin O–permeabilized T2 cells transfected with rat TAP1+2ᵃ (T2/TAP1+2) (*black bars*) and, as a control, T2 cells (*open bars*) as indicated. Translocated peptides were isolated with Con A–Sepharose, quantitated, and expressed as the percentage of input peptide. The size of the respective input peptides is indicated on the horizontal axis. The peptides are, respectively, #49, #50, #53, #54, SN12, SN12A1, #55, SN11, #88, #89, and #90 (*top*) and #146, #147, #148, #149 and #150 (*bottom*). For sequence, see Materials and Methods.
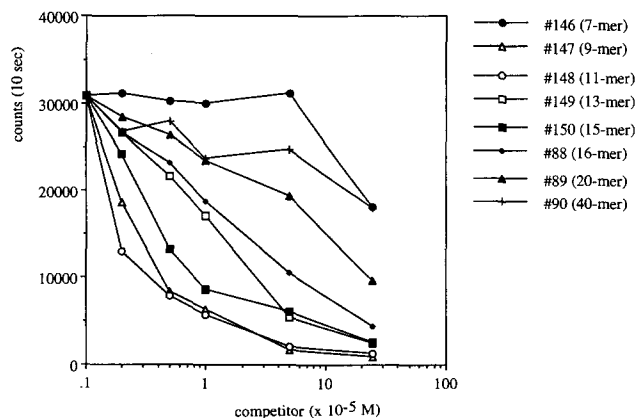

Figure 2. Competition for translocation of a 9-mer model peptide with peptides of different length. The iodinated 9-mer peptide #147 (sequence TVDNKTRAY) was translocated for 5 min in washed streptolysin O–permeabilized T2/TAP1+2 cells in the presence of different concentrations of competitor peptides of different size. The glycosylated peptides were recovered by Con A–Sepharose and quantitated. The 9- and 11-mer peptide competed most efficiently. Longer peptides as well as smaller peptides competed less efficiently for translocation of the 9-mer input peptide.

containing the N-linked glycosylation consensus sequence (Asn-X-Thr/Ser) were added to streptolysin O permeabilized cells in the presence of ATP. Translocation of such peptides into the ER results in the addition of a N-linked glycan. The resulting glycosylated peptide can be isolated with Con A–Sepharose beads and further analyzed. We used the mutant cell line T2 that has a deletion of the TAP genes, and compared them with T2 cells reconstituted with cDNAs encoding rat TAP1 and TAP2ᵃ (T2/TAP1+2) (24). Thus, any difference in the recovery of translocated peptides in T2 and T2/TAP1+2 cells can only be due to expression of TAP1 and TAP2ᵃ.

A number of model peptides of different size and sequence were synthesized (cf. Materials and Methods) and radioiodinated. The amount of translocated peptide was determined by γ counting (Fig. 1). Whereas none of the peptides was translocated in streptolysin O–permeabilized T2 cells, peptide translocation was observed in permeabilized T2/TAP1+2 cells for all peptides except the 4 and 5 mer. We conclude that TAP does not select peptides with a size below six amino acids.

The Relative Affinity of Different Peptide Lengths for TAP. The outcome of the translocation experiment shown in Fig.

1 is influenced by the relative affinity of the peptides for TAP, the transit time, and their stability in the cytosol and the ER. To determine the relative affinities for TAP of peptides of different length, T2/TAP1+2 cells were permeabilized, the cytosol was removed by washing, and translocation of the 9-mer peptide #147 (sequence TVDNKTRAY) was inhibited with unlabeled peptides of different size but similar sequence (see Materials and Methods). To reduce any effect of proteolysis of the input and competitor peptides, the translocation assay was performed for only 5 min. The translocated peptides were recovered with Con A–Sepharose and quantitated (Fig. 2). The 9- and 11-mer peptide competed most efficiently (50% inhibition at ∿2 μM). 50% inhibition for translocation with the 8-mer peptide #53 was found at ∿10 μM and for the 10-mer peptide #55 between 1 and 5 μM (not shown). With increasing length, the peptides inhibited less efficiently with the exception that the 15 mer competed somewhat better than the 13-mer peptide. The 20-mer peptide competed ∿50 times less well than the 9 and 11 mer, and the 40 mer was an even poorer competitor. Also, the 7-mer peptide #146 competed very inefficiently, although a small amount of the 7-mer peptide was actually translocated, as indicated by the experiments in which directly labeled peptide was used (Fig. 1 *B*). Thus, peptides with an optimal length for binding to class I molecules (8–11 amino acids) inhibited TAP-dependent transport most effectively, suggesting that these peptides bound TAP with the highest affinities.

Translocation of Degradation Intermediates. The observations that radiolabeled glycopeptides were isolated with Con A–Sepharose after TAP-dependent translocation of a 20- or 40-mer iodinated peptide (Fig. 1) and that these peptides have a low affinity for TAP (Fig. 2) did not exclude that the 20- or 40-mer peptides were proteolytically truncated before being
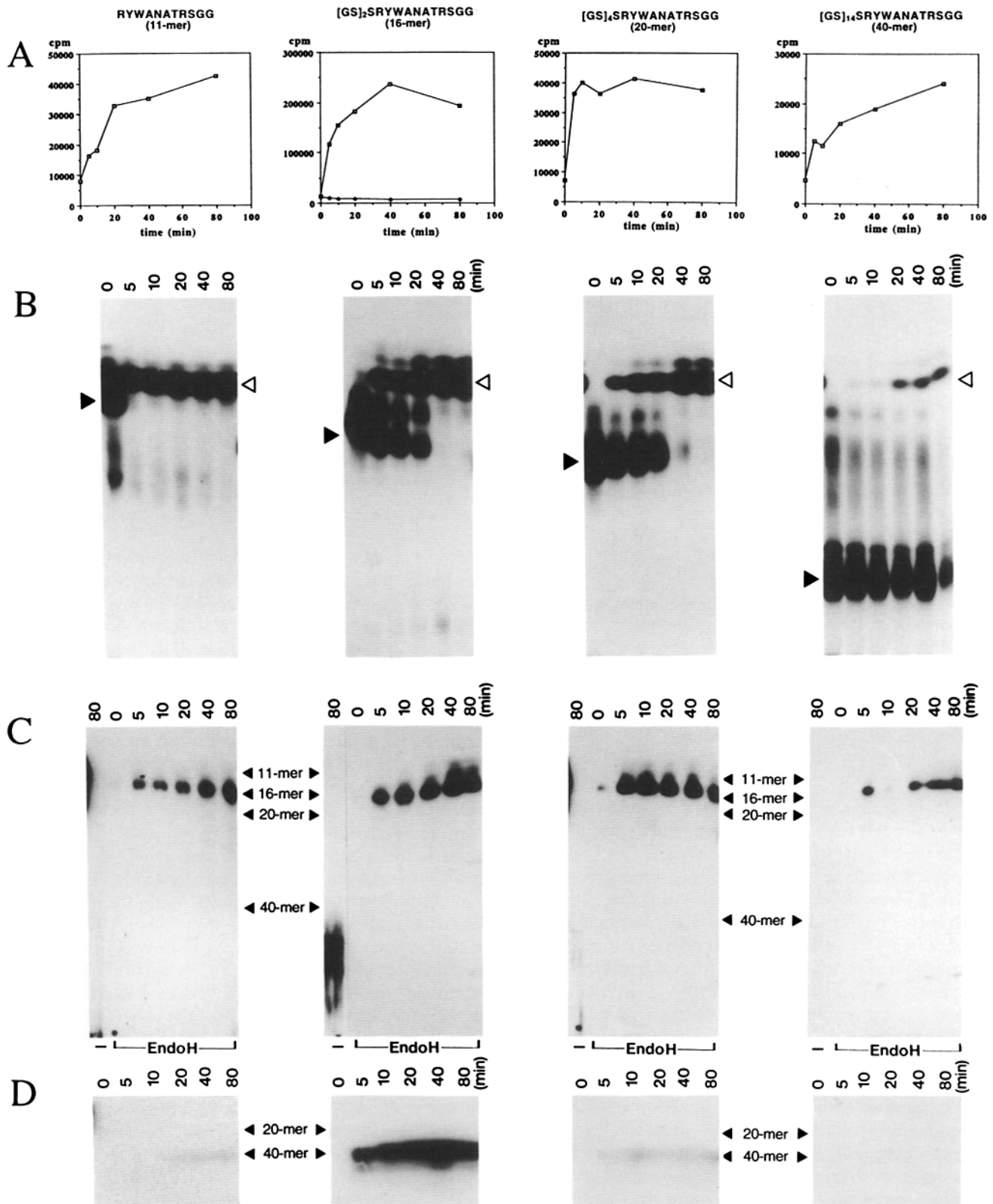
Figure 3. Peptide size selection by TAP. (*A*) Kinetics of translocation of the 11-mer peptide SN11 and the 16 (#88), 20- (#89) and 40-mer (#90) derivatives from SN11 generated by a NH2-terminal repeat of S and G residues, as indicated above the figure. The corresponding experiments shown in Fig. 3 (*B–D*) are placed below the respective figures. The peptides were translocated for different periods in streptolysin O–permeabilized T2/ TAP1+2 cells in the presence of ATP. As a control, the 16-mer peptide was translocated in the absence of ATP (*closed symbols*). At the respective time points, cells were lysed and the translocated peptides were isolated with Con A–Sepharose. For all four peptides, transport is rapid within the first

transported. To examine the importance of peptide degradation in the cytosol in our experiments, we simultaneously measured translocation and degradation of the 11-mer model peptide RYWANATRSGG and the same peptide elongated at the NH$_2$ terminus by a repeat of S and G to a 16, 20, and 40 mer, respectively. The peptides were iodinated and the kinetics of translocation was determined (Fig. 3 A; the sequence of the peptides is shown on top). The highest rates of translocation were observed during the first 10–20 min. Note that translocation again was most efficient with the 16-mer peptide and least efficient with the 40-mer peptide (cf. Fig. 1).

To follow the fate of the input peptide, an aliquot of the reaction mixture (containing permeabilized cells, ATP, and input peptide) was taken at each time point and analyzed by TLC (Fig. 3 B). The closed triangles show the position of the input peptide and the open triangles show the position of the major breakdown product, which is most probably iodotyrosine. The rate of degradation of the input peptide was inversely correlated with the length of the peptide: the 11-mer peptide was degraded rapidly and the 40-mer peptide slowly.

Next we analyzed the glycosylated (and thus translocated) peptide on TLC (Fig. 3 C). The positions of the different input peptides are indicated by their size. The glycosylated peptides migrated as a smear on TLC (lanes "–") because of the heterogeneity of glycosylation. The N-linked glycan (except for the inner GlcNAc) was removed with Endo H and the deglycosylated peptides were again analyzed (lanes Endo H). Iodinated peptides migrating at a similar position were observed after translocation of all the four different input peptides. This position corresponded to a migration between the 11- and the 16-mer input peptides which were run as standards. Since TLC mainly separates according to hydrophobicity but not by size, this did not imply that the transported, deglycosylated peptide had a length somewhere between 11 and 16 amino acids. However, the fact that the input and the translocated peptide migrated at a different position on TLC suggested that TAP selects for peptides of a defined length that can be generated by degradation of longer peptides. The glycosylated peptides were also analyzed by Tricine SDS-PAGE, which allows separation of small molecules mainly according to size (Fig. 3 D). The position of the 20- and 40-mer input peptide is indicated. A band migrating approximately at the position of the 40-mer input peptide with an approximate molecular weight of ~3.4 kD is observed for the 11-, 16-, and 20-mer input peptide (we did not recover enough translocated 40-mer peptide to allow visualization). The N-linked

glycan has a molecular mass of ~2.4 kD. Thus, the molecular mass of the peptide core can be estimated to ~1 kD, corresponding to about 9–11 amino acids. Our data suggest that the translocated peptides have a similar molecular weight for the three input peptides, implying that short peptides of defined size are selected by TAP.

Peptides Containing an N-linked Glycan Are Stable in the ER. Peptide translocation approached a steady state after some 20 min (reference 16; Fig. 3 A). If this was only caused by shortage of the input peptide due to degradation, this would imply that the translocated and thus glycosylated peptide is stable in the ER. To examine the stability of glycosylated peptides in the ER, further import of peptides was inhibited by depleting ATP with hexokinase (HK) and glucose. This treatment resulted in almost complete inhibition (>85%) of peptide translocation within 10 min (Fig. 4 A). To address the question of glycopeptide stability in the ER, peptide translocation in T2/TAP-1+2 was allowed to proceed for 10 min in the presence of ATP. Thereafter ATP was depleted (or not) with HK and glucose and the cells were further incubated at 37°C for various times. Translocation was not complete after 10 min because undepleted cells consistently contained larger amounts of glycosylated peptide than ATP-depleted samples (Fig. 4 B). Furthermore, there was no decrease in the amount of recovered glycopeptide for as long as 80 min after ATP depletion. This indicated that the peptide, once imported in the ER and glycosylated, remained stable. To show that the peptides were indeed not further modified in the ER, we analyzed the Con A–isolated peptides after Endo H treatment by TLC (Fig. 4 B). The peptides isolated at the different time points migrated at identical positions, confirming that the peptides were not further modified in the ER. This was true for all of the four peptides studied (#72, #88, #78, and SN12A1).

The Size of the Translocated Peptides. The translocated peptides analyzed in Fig. 4 B were further characterized by establishing the position of the iodotyrosine (Y). This was done by determining the number of Edman degradation steps required to release the iodine label (Fig. 5 A). This allowed us to determine the number of amino acids located on the NH$_2$-terminal side of the iodinated Tyr (Y) but did not provide information on the exact location of the COOH terminus with the peptides used here. Using radiolabeled peptide #72 (sequence RYWANATRSY) in a translocation assay, two major transported glycopeptides were recovered (Fig. 4 B), which were extracted separately from TLC plates and subjected to Edman degradation. This showed the upper band on the chromatogram to contain a peptide with Tyr at posi-

10 min and then levels off. No peptide translocation is observed in the absence of ATP. (B) Degradation of the input peptides. Samples were taken from the reaction mixture at the time points indicated above the figure and analyzed on TLC. (Closed arrowhead) The position of the input peptide; (open arrowhead) the position of the major breakdown product. The input 11-mer peptide is degraded very rapidly, the 16- and 20-mer peptides are degraded after some 20 min, and the input 40-mer peptide is degraded slowly. (C) TLC analysis of the translocated peptides. The glycosylated peptides, quantitated in Fig. 3 A, are released from Con A–Sepharose after transport for the indicated periods and analyzed by TLC either untreated (lanes "–") or after Endo H treatment (lanes Endo H). The positions of the 11-, 16-, 20-, and 40-mer input peptide are indicated. Whereas the glycosylated peptides migrated as a smear on TLC plates, after deglycosylation with Endo H the translocated peptides derived from the 11, 16, 20, and 40 mer all migrated at a similar position intermediate between the 11- and 16-mer input peptides used as marker. (D) Analysis of the translocated peptides by Tricine SDS-PAGE. The translocated peptides were released from Con A–Sepharose and analyzed by Tricine SDS-PAGE. The translocated peptides migrate at the position of the (unglycosylated) 40-mer input peptide used as size standard along with the 20 mer.
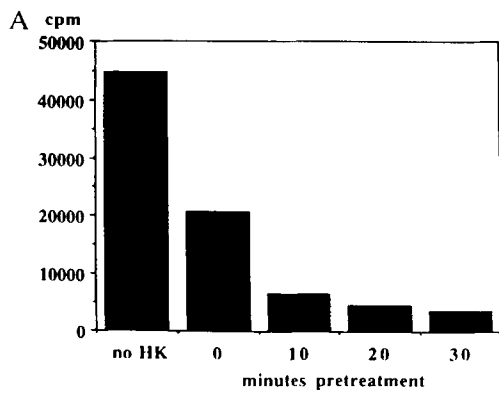
A cpm

Figure 4. Glycosylated peptides are stable in the ER. (*A*) Inhibition of peptide translocation by depletion of ATP with HK and glucose. To determine the efficiency of inhibition of peptide translocation by depletion of ATP, peptide #72 (sequence RYWANATRSY) was added to streptolysin O–permeabilized T2/TAP1+2 cells for 10 min either in the presence of ATP alone (bar *no HK*), or after different periods of pre-incubation with ATP, HK, and glc, as indicated. Translocated peptides were isolated with Con A–Sepharose and quantitated. Pre-incubation for 10 min with HK/glc significantly inhibited ATP-dependent peptide translocation. (*B*) The stability of translocated peptides. Peptides #72, #88, SN12A1, or #78 (sequence indicated above the figure) were translocated in streptolysin O–permeabilized T2/TAP1+2 cells either in the presence (*closed squares*) or absence (*closed triangles*) of ATP (*top*). In parallel samples, ATP was depleted by addition of HK/glc after a 10-min translocation in the presence of ATP (*open circles*). Cells were lysed after different periods and the translocated peptides were recovered with Con A–Sepharose. Peptide translocation was ATP dependent and depletion of ATP resulted in a decreased recovery of translocated peptides. The iodotyrosine label was not removed from the translocated peptides. The translocated peptides were further analyzed by TLC after removal of the N-linked glycan with Endo H (*bottom*). The input peptide is shown for comparison. No detectable modifications of the translocated peptide were observed by TLC indicating that the glycosylated peptides remained stable in the ER. Note that input peptide #72 appeared as two distinct species in the ER.
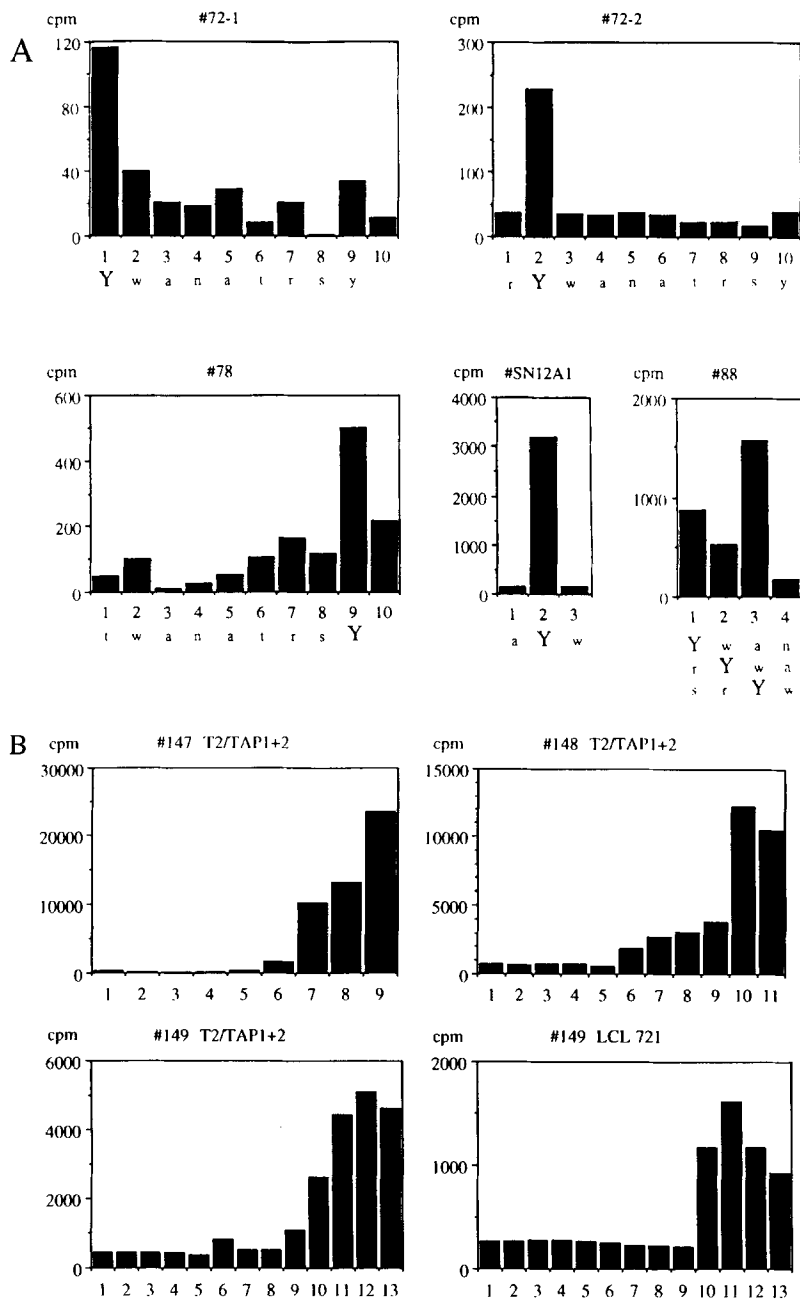
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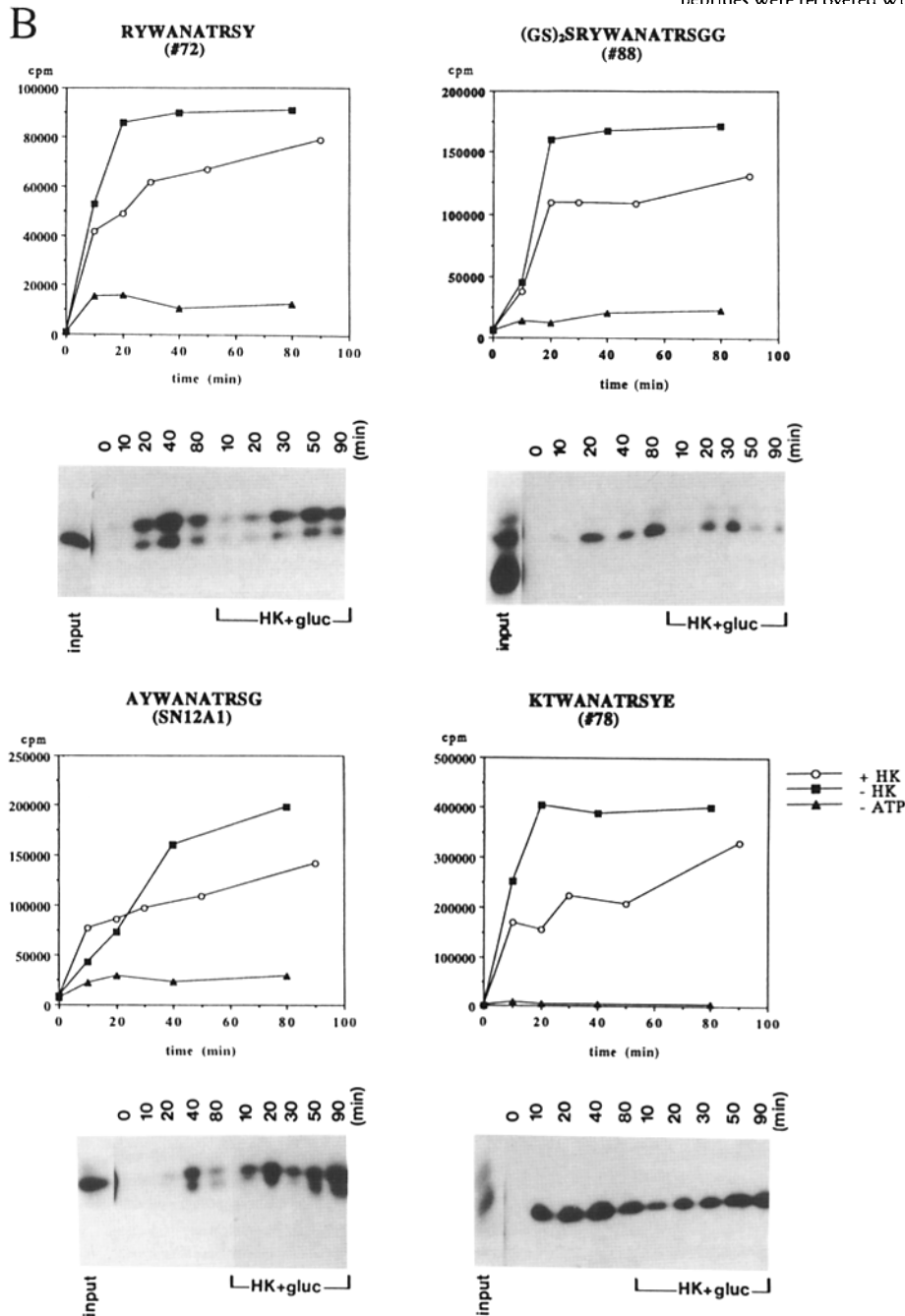
Figure 5. Sequence analysis of the translocated peptides. (*A*) Analysis of the translocated peptides #72, SN12A, #78, and #88, shown in Fig. 4 *B*. The peptides were isolated from the TLC plate and the number of Edman degradation steps required to release the label (corresponding to iodotyrosine [Y]) was determined. The number of Edman degradation steps is indicated on the *x*-axis and the amount of radioactivity released in every step on the *y*-axis. The predicted sequence is shown. The upper band of the doublet observed after translocation of peptide #72 starts with Y at position 2 of the input peptide (*#72-1*), and the lower band at R at position 1 of the input peptide (#72-2). The translocated peptide derived from the 16-mer peptide #88 starts with S, R, or Y at, respectively, position 5, 6, or 7 of the peptide. The predominant peptide recovered after translocation of input peptide #78 starts with T at position 2. Translocated peptide SN12A1 starts with A at position 1, like the input peptide. (*B*) Size of the translocated peptides. The peptides #147 (9 mer; sequence TVDNKTRAY), #148 (11 mer; sequence ETVDNKTFRAY), and #149 (13 mer; sequence IETVDNKTSFRAY) were added to streptolysin O–permeabilized T2/TAP1+2 or LCL 721 cells (as indicated) and the translocated peptides were recovered with Con A–Sepharose. Because the peptides contain a COOH-terminal radioiodinated Y residue, the number of Edman degradation steps required to release the label is directly correlated to the length of the translocated peptide. The number of Edman degradation steps is indicated on the *x*-axis; the radioactivity released in the respective cycles on the *y*-axis. The rat and human peptide transporters translocate peptides with a size of 7–13 amino acids.

tion 1, whereas the lower band had Tyr at position 2 and presumably was unmodified at the NH₂ terminus (Fig. 5 *A*). By a similar analysis, the translocated glycopeptide species derived from peptide #88 ([GS]₂SRYWANATRSGG) contained Tyr mainly at position 1 to 3. The SN12A1 (sequence AYWANATRSG) derived translocation product contained Tyr at position 2, whereas most of the product of the 11-mer peptide #78 (sequence KTWANATRSYE) had Tyr at position 9 (Fig. 5 *A*). The latter peptide could not have been shortened at the COOH terminus by more than one amino acid.

To establish the exact size of the translocated peptides, the

9-mer peptide #147, the 11-mer peptide #148, and the 13-mer peptide #149, all containing a COOH-terminal Y, were translocated in T2/TAP1+2ᵃ cells and the number of Edman degradation steps required to release the iodine label (now corresponding to the COOH-terminal amino acid) was determined (Fig. 5 *B*). The peptide #147 was mainly translocated as a 9-mer, but also 8- and 7-mer peptides were recovered from the ER. Mainly a 10- and a 11-mer peptide were translocated after addition of peptide #148, but minor amounts of smaller degradation products were also found. Peptides ranging from 9 to 13 amino acids derived from peptide #149 were translocated. To establish whether the human peptide
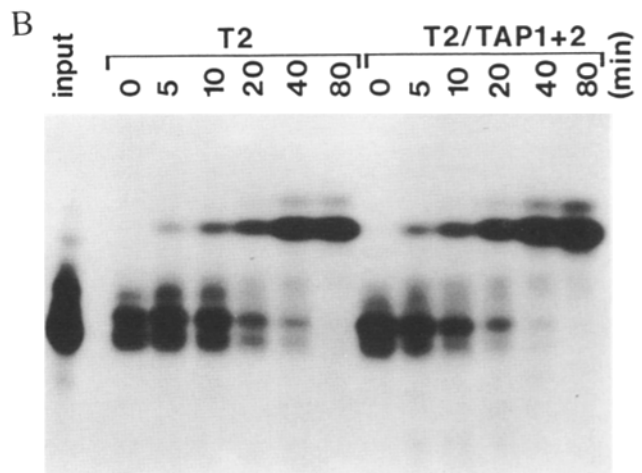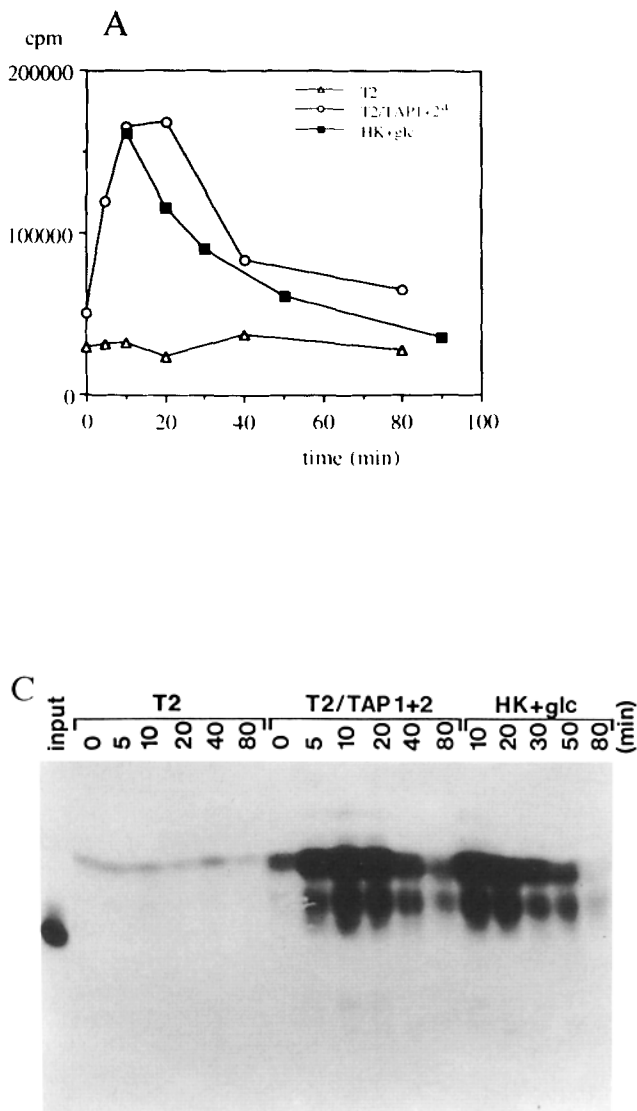
Figure 6. Peptide degradation in the ER. (A) Stability of unglycosylated peptide #88 (sequence GSGSSRYWANATRSGG) in the ER. N-linked glycosylation was inhibited with tunicamycin and peptide #88 was translocated in streptolysin O–permeabilized T2 (open triangles) or T2/TAP1+2 (open circles) cells for different periods in the presence of ATP. Alternatively, further peptide translocation was inhibited after 10 min by addition of HK and glc to permeabilized T2/TAP1+2 cells (closed squares). At the respective time points, cells were transferred to 0°C, washed extensively, and the amount of radioactivity retained was quantitated. Peptide #88 is efficiently translocated by TAP during the first 10 min, followed by release of the translocated peptides after 20 min. (B) Degradation of the input peptide #88. Like Fig. 3 B, samples of the input peptide were analyzed by TLC after different periods of incubation with streptolysin O–permeabilized T2 or T2/TAP1+2 cells, as indicated. The input peptide #88 is rapidly degraded at similar rates in T2 and T2/TAP1+2 cells. (C) Analysis of the translocated peptides by TLC. The cells containing the associated peptide, as quantitated in A, were extracted with phenol, analyzed by TLC, and the radiolabeled peptide was detected by autoradiography. The corresponding time points and conditions are indicated. A number of peptides were exclusively retained in T2/TAP1+2 cells. Their appearance follows the kinetics shown in A, and they migrate differently from the input peptide #88 and the major degradation product (Fig. 6 B). ATP depletion with HK and glc does not inhibit peptide release. Note the minor radiolabeled species associated with T2/TAP1+2 cells.

transporter selects for peptides in a similar fashion as rat TAP1+2ᵃ, the 13-mer peptide #149 was also translocated in streptolysin O–permeabilized LCL 721 cells (Fig. 5 B, #149 LCL 721). The peptides derived from peptide #149 contained 10–13 amino acids as concluded from the Edman degradation of the transported peptide mixture in LCL 721.

Non-glycosylated Peptides Are Unstable in the ER. Glycosylated peptides are stable in the ER (see Fig. 4 B). This may be due either to the absence of proteolytic activity in the ER, or to protection by the N-linked glycan against proteolysis or re-export. We studied the fate of the translocated peptide #88 in T2 and T2/TAP1+2 cells that were treated with tunicamycin to inhibit N-linked glycosylation. Since now the translocated peptide could not be recovered by Con A–Sepharose through its N-linked glycan, peptide translocation was followed by extensive washing of the streptolysin O–permeabilized cells. Peptides in the intact ER will be protected against removal by washing. After washing, the cells were extracted with phenol to denature all proteins and the radioactivity

recovered in the phenol phase was quantitated (Fig. 6 A). We determined beforehand that the majority (~80–90%) of input peptide will partition into the phenol phase. Again, any difference between T2 and T2/TAP1+2 cells in the amount of radioiodinated peptide retained can only be attributed to TAP, which will translocate the peptides into the ER. In T2 cells, similar amounts of background radioactivity were recovered at each time point. Expression of TAP resulted in a rapid increase in the amount of radioactivity recovered during the first 10 min, similar to the increase observed for cells that were not treated with tunicamycin (cf. Figs. 3 A and 4 B). However, this initial increase was now followed by a rapid drop in radioactivity resistant to washing, suggesting rapid release of the peptide (or fragments thereof) from the ER. Also, inhibition of further translocation by enzymatic depletion of ATP after translocation for 10 min resulted in a rapid decrease in the amount of nonglycosylated peptide retained in cells (Fig. 6 A).

We then analyzed the fate of the input peptide (Fig. 6 B)

and the translocated peptide (Fig. 6 C) by TLC. The input peptide was rapidly and in a similar fashion degraded in both T2 and T2/TAP1+2 cells (see also Fig. 3 B). The breakdown intermediates were not identified. After extensive washing, a background band was still associated with permeabilized T2 cells (Fig. 6 C). However, a number of bands retained in permeabilized T2/TAP1+2 cells after extensive washing appeared during the first 10 min and disappeared at later time points. Note the appearance of minor radiolabeled degradation species. Addition of HK and glucose (glc) after a 10-min translocation of peptide #88 also showed a clear decrease in the amount of isolated peptide. The half-life of the non-glycosylated peptide fragment #88 in the ER can be estimated to be ∿20 min.

Discussion

MHC class I molecules present peptides that are derived from nuclear or cytosolic antigens to CD8^{+} T cells. The resolution of the three-dimensional structure of class I molecules associated with peptide showed that the peptide is deeply embedded in the binding groove (31, 32), explaining the observation that peptide is essential for the stability of class I molecules (3). These nuclear/cytosolic peptides are trapped by class I H chain/β_2-microglobulin heterodimers in the lumen of the ER (3, 4, 6) which is preceded by translocation of the peptides from the cytosol to the lumen of the ER by the TAP heterodimer (16, 26, 27). TAP shows substrate specificity (33) and selects peptides with proper NH_2- and COOH-terminal residues (34, 35). However, it is still unclear how antigenic peptides are generated, in what form they are translocated and what their fate is in the lumen of the ER.

We show that intact 16-mer or longer model peptides were not or were inefficiently translocated by TAP. Instead, we found translocated peptides derived from the 16-mer input peptide #88 that contained 12 or fewer amino acids. This indicates that TAP selects for the size of the peptide. The upper limit is not exactly defined here but we show that a 13-mer model peptide (#149) was translocated without modifications, whereas the 16-mer (#88) peptide required prior trimming. It was recently shown that TAP-dependent peptide translocation can be inhibited by peptides of different length (16, 31, 32), but these studies were not fully conclusive. Since the fate of the input peptide was not followed, it was unclear whether competition was due to the input peptide or a degradation fragment thereof. Furthermore, since competition for translocation does not imply that the competitor is translocated, the actual size of the translocated peptides could not be established in these studies.

We determined the size of the translocated peptide by Edman degradation. The smallest peptides we found translocated contain 6–7 amino acids whereas the longest contained 13 amino acids. Since peptides of 7 or 12–13 amino acids are unlikely to associate with class I molecules with high affinity (although there may be exceptions), this indicates that the size selection by TAP does not accurately meet the requirements for optimal binding to class I molecules.

Nevertheless, it is striking that the size range of peptides found associated with class I molecules (8–11 amino acids; reference 1) is covered by the size of peptides selected by TAP. However, longer peptides do compete for translocation of a 9-mer model peptide but the relative affinity decreases with size. Probably, peptides longer than 13 amino acids can be translocated, but with lower efficiency. As a result, these longer peptides will rather be degraded in the cytosol and their degradation intermediates of optimal size (8–11 amino acids) will be preferably selected by TAP because these intermediates have the highest (relative) affinity for TAP (see Fig. 3). This, as we show, does not exclude the possibility that shorter or longer peptides enter the ER. Furthermore, the amount of peptides that ultimately enter the ER will be determined by the sequence as well as the relative concentration of these peptides in the cytosol. Other peptide transporters with ATP-binding cassettes have been described but their size selection is different. The yeast protein STE6 transports a lipid-modified 12-mer peptide, the a-factor, across the plasma membrane (36). The bacterial oligopeptide permease Opp, however, transports peptides of up to five amino acids in length (37).

In the lumen of the ER, MHC class I heterodimers associate with peptides of 8–11 amino acids (1). Since slightly longer peptides apparently can be translocated, these peptides might require trimming before being able to bind tightly to class I molecules. In the assay used in this study, we followed N-linked glycosylation of peptides as a marker for appearance in the lumen of the ER. We show that the N-linked glycan protects peptide against degradation in the ER. However, most naturally occurring peptides do not obtain an N-linked glycan in the ER and we show that these peptides are rapidly degraded and released from the ER. The substrate specificity of this peptide export mechanism is unknown and their prime function may be to remove signal sequence fragments and peptides that do not associate with class I molecules, from the ER lumen. Thus, class I molecules, the ER-degradation machinery, and the mechanism that releases peptides from the ER, compete for peptides in the ER. If a peptide fails to bind to class I molecules, it will be rapidly exported from the ER and degraded outside. This explains why Falk et al. (38) were unable to detect a peptide fraction in cells other than the class I–bound peptides. Due to rapid degradation, a stable pool of peptides is apparently not available in the ER or the cytosol. However, ER-located accessory molecules, like BiP (39) and grp94 (40), may bind some peptide and thereby protect them against further degradation in the ER and thus serve as a peptide reservoir. Whether these molecules are involved in the loading of class I molecules with peptide remains to be established.

We have followed the early processes involved in MHC class I–restricted antigen presentation using streptolysin O–permeabilized cells. We show that input peptides are rapidly degraded and that TAP concomitantly selects for peptide fragments with a size range that covers the length of peptides associated with class I molecules. Peptides that do not bind to class I molecules are rapidly released from the ER.

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