Human Transporters Associated with Antigen Processing (TAPs) Select Epitope Precursor Peptides for Processing in the Endoplasmic Reticulum and Presentation to T Cells

By Grégoire Lauvau,* Kazuhiro Kakimi,‡ Gabriele Niedermann,§ Marina Ostankovitch,‖ Patricia Yotnda,¶ Hüseyin Firat,¶ Francis V. Chisari,‡ and Peter M. van Endert*

*From the *Institut National de la Santé et Recherche Medicale (INSERM) U25, Hôpital Necker, 75015 Paris, France; ‡Scripps Research Institute, La Jolla, California 92037; §Max-Planck-Institut für Immunbiologie, D-79108 Freiburg, Germany; ‖INSERM U455, ICGM, 75014 Paris, France; and ¶Institut Pasteur, Département SIDA-Rétrovirus, Unité d'Immunité Cellulaire Antivirale, 75724 Paris cedex 15, France*

Summary

Antigen presentation by major histocompatibility complex (MHC) class I molecules requires peptide supply by the transporters associated with antigen processing (TAPs), which select substrates in a species- and, in the rat, allele-specific manner. Conflicts between TAPs and MHC preferences for COOH-terminal peptide residues in rodent cells strongly reduce the efficiency of MHC class I antigen presentation. Although human TAP is relatively permissive, some peptide ligands for human histocompatibility leukocyte antigen class I molecules are known to possess very low TAP affinities; the significance of these in vitro findings for cellular antigen presentation is not known. We studied two naturally immunodominant viral epitopes presented by HLA-A2 that display very low affinities for human TAP. Low TAP affinities preclude minimal epitope access to the endoplasmic reticulum (ER) and assembly with HLA-A2 in vitro, as well as presentation by minigene-expressing cells to cytotoxic T lymphocytes. However, NH_2-terminally but not COOH-terminally extended epitope variants with higher TAP affinities assemble in vitro and are presented to cytotoxic T lymphocytes with high efficiency. Thus, human TAP can influence epitope selection and restrict access to the ER to epitope precursors. Analysis of TAP affinities of a panel of viral epitopes suggests that TAP selection of precursors may be a common phenomenon for HLA-A2–presented epitopes. We also analyzed HLA-A2–eluted peptides from minigene-expressing cells and show that an NH_2-terminally extended variant with low A2 binding affinity undergoes ER processing, whereas another with high affinity is presented unmodified. Therefore, the previously reported aminopeptidase activity in the ER can also act on TAP-translocated peptides.

Key words: antigen presentation • ABC transporters • immunodominant epitopes • HLA class I • aminopeptidases

Human histocompatibility leukocyte antigen class I (HCI)[1] molecules present short peptides with a length of 8–10 residues to CTLs. Most of these peptides are derived from degradation of cellular proteins by the cytosolic proteasome complex and translocated into the endoplasmic reticulum (ER) by the heterodimeric complex of the transporters associated with antigen processing (TAPs) (for review see references 1–3). This pump is inserted into the membranes of ER and cis-Golgi compartments and delivers peptides to empty HCI/β2-microglobulin (β_2m) dimers that associate with TAP with the help of the 48-kD protein tapasin, whose function remains unclear (4).

An important unresolved question concerns the contribution of the different steps in antigen processing to selection of immunodominant epitopes. Studies of the relationship between peptide affinity for MHC class I molecules and efficiency of presentation have demonstrated that high MHC binding affinity generally is a necessary but insufficient prerequisite for immunogenicity; this suggests additional roles for antigen processing and/or the T cell repertoire (5, 6). Selective TAP transport is one of the principal candidate

[1]*Abbreviations used in this paper:* β_2m, β2-microglobulin; BFA, brefeldin A; Ct, COOH-terminal; ER, endoplasmic reticulum; HBV, hepatitis B virus; HCI, HLA class I; HCV, hepatitis C virus; MOI, multiplicity of infection; Nt, NH_2-terminal; TAPs, transporters associated with antigen processing.

 J. Exp. Med. © The Rockefeller University Press • 0022-1007/99/11/1227/13 $5.00
Volume 190, Number 9, November 1, 1999 1227–1239
http://www.jem.org

mechanisms for epitope selection during processing. The principles of peptide selection by rodent and human TAP transporters have been studied in detail using two in vitro assays (7, 8). TAP transporters display species- and (in the rat) allele-specific substrate selectivities, the level of which is highest for murine TAP and rat TAP1–TAP2^b complexes and significantly lower for human TAP; TAP selects substrates according to the COOH-terminal (Ct) residue followed by the three NH_2-terminal (Nt) residues (7–9).

The significant potential impact of TAP selectivity on antigen presentation was first demonstrated by Powis et al., who studied rat cells expressing TAP transporters and MHC class I molecules with incompatible preferences for Ct peptide residues (10). This conflict impairs efficiency of antigen presentation so significantly that incompatible TAP/MHC combinations do not occur naturally in rat strains that have not been selected for recombined MHC regions (11). Peptide selection by murine TAP can also affect antigen presentation, as shown recently by Yellen-Shaw et al. (12); in this case, TAP selection was unfavorable for peptides with Ct residues that are not preferred by murine MHC class I molecules, confirming the absence of conflicting TAP and MHC preferences in naturally selected rodent strains. Interestingly, detection of peptide selection by TAP required limitation of minigene expression, demonstrating that low TAP affinities could be overcome by abundant peptide levels in the cytosol (12).

Human TAP transporters are more permissive than murine or rat TAP-B transporters and accept peptides with all Ct residues generally found in HCI-binding peptides, albeit with different efficiencies (9, 13). Nevertheless, variations of the three Nt and the Ct peptide residues that together determine peptide affinity for TAP can cause dramatic differences in peptide affinity for TAP (8, 9). As human TAP preferences are much more compatible with those of some HCI alleles such as HLA-B27 than those of others such as HLA-A2, potential ligands for some HCI molecules frequently possess low TAP affinities (14). The biological consequences of this phenomenon, and more generally those of human TAP selectivity, have so far not been investigated. Several scenarios may account for efficient HCI-mediated presentation of peptides with very low TAP affinities and for efficient antigen presentation by HCI alleles that prefer ligands with low TAP affinities. First, high cytosolic concentrations of peptides with low TAP affinities may overcome inefficient TAP transport, as indeed has been demonstrated in the murine experimental system cited above (12). Second, HCI alleles with poorly TAP-adapted peptide preferences may present a smaller array of epitopes due to inefficient TAP-mediated ER access for numerous potential ligands. Finally, these alleles may frequently present peptides that enter the ER as precursors with higher TAP affinities and are then processed to the final epitope within the ER. We and others have previously found circumstantial in vitro evidence consistent with the latter hypothesis: in TAP binding (8) as well as transport (15) assays, extended precursors of some epitopes with low TAP affinities were found to possess higher TAP affinities. However, neither lack of epitope presentation due to low affinity for human TAP nor reconstitution of epitope presentation due to ER entry of precursors with higher TAP affinities has so far been demonstrated in cellular assays. Given the exquisite sensitivity of epitope recognition by CTLs and the phenomenon of a relatively low threshold for efficient antigen processing (16), it can be argued that even extremely inefficient TAP transport, as observed in in vitro assays, may be sufficient for epitope presentation (17).

The hypothesis of epitope precursor transport as a rescue pathway for poor TAP/MHC coordination or as a way of increasing the variety of HCI-presented peptides implies an efficient mechanism for peptide adaptation to MHC class I binding requirements in the ER. Although ER peptidases involved in maturation of antigenic peptides have not been identified, available evidence indicates that antigenic peptides can be modified by an aminopeptidase activity, whereas COOH terminals cannot be modified in the ER (10, 18, 19). This conclusion is based on experiments in which expression of signal peptide–coupled protein fragments or epitope precursors in TAP-deficient APCs resulted in efficient release and presentation of epitopes (19–21). Epitopes can also be liberated by an aminopeptidase activity from the COOH terminus of the type 2 transmembrane protein Jaw 1 (22). Nt trimming of epitopes in the ER is consistent with the capacity of TAP to transport long peptides (8, 23) and with observations suggesting that the proteasome very often generates the COOH terminals and frequently, but not always, the NH_2 terminals of MHC class I–presented epitopes (24).

Even though there is clear evidence for an aminopeptidase activity in the ER, some observations suggest that its efficiency may be limited or that it may not act on all peptide–MHC complexes. A subset of cell surface HLA-B27 molecules present long peptides unlikely to have been subjected to a peptide maturation mechanism (25). Moreover, a recent analysis of peptide assembly with HLA-B27 in tapasin-deficient cells suggests that tapasin may be important for optimization of HCI-presented peptides (26). Because evidence for Nt epitope maturation in the ER was obtained using signal peptide–coupled epitopes and because it is not known whether such epitopes assemble with HCI molecules in tapasin-associated loading complexes, processing of signal peptide–coupled epitopes may not fully reflect that of TAP-transported peptides. The finding that most signal sequence–derived peptides eluted from HLA-A2 in TAP-deficient cells are longer than the standard 8–10 residues (27) is compatible with the hypothesis that HCI ligands from this alternative source may not be subjected to the same editing mechanisms as TAP-supplied peptides. Thus, although most authors agree that available evidence renders Nt ER processing of TAP-transported peptides likely (1, 3, 18), it is important to provide direct evidence for this suggestion; no example of ER processing of a TAP-transported peptide has been provided as yet.

We have studied biological effects of the selectivity of human TAP and potential peptide processing in the ER in the models of two naturally immunodominant viral epitopes presented by HLA-A2. We find that the selectivity of human

TAP complexes effectively precludes presentation of these short epitopes expressed as minigenes in the cytosol and favors ER access of longer precursor peptides. As revealed by analysis of HLA-A2–eluted peptides, TAP-transported precursors with low HCI binding affinity can undergo rapid and complete processing in the ER.

Materials and Methods

Peptides, Antibodies, and Cell Lines

The panel of HLA-A2 binding peptides derived from hepatitis B virus (HBV) and hepatitis C virus (HCV) sequences has been described previously (28, 29). Peptide HBV pol 575-83 (FLLSLGIHL) and its variant 573-83, HBV core 18-27 (FLPSDFFPSV) and its variants 17-27 and 18-28, reporter peptides HLTV-I tax 11-19 (LLFGYPVYV), HIV gag 77-85 (SLYNTVATL), R-9-L (RRYNASTEL), R-10-T (RYWANATRST), S-9-R (SRYWAIRTR), S-11-R (SRYWNATIRTR), and F-10-V (FLPSDYFPSV) were all obtained >96% pure from Genosys. HBV epitope variants pol 575-87, 573-87, and core 17-28 were synthesized with >95% purity by the peptide synthesis facility of the Scripps Research Institute.

Hybridomas BB7.2 with specificity for HLA-A2 and IVD12 recognizing HLA-DQ were purchased from American Type Culture Collection (ATCC). HLA-A2–expressing B cells used in this study were provided by the Centre pour l'Etude du Polymorphisme Humain (Paris, France) or A. Toubert (Hôpital St. Louis, Paris, France) and included homozygous lines Jesthom and JY and mutant line T2. RMA-S cells transfected with "HHD" HLA-A2/K^d molecules were grown with 1 mg/ml gentamycin and 10^{-5} M β-ME as described (30).

TAP Peptide Binding Assay

Peptide binding to human TAP complexes was measured as described (8) using reporter peptide R-9-L at 300 nM.

Translocation Assays

Human B cell lines were washed once in translocation buffer (130 mM KCl, 10 mM NaCl, 1 mM $CaCl_2$, 2 mM EGTA, 2 mM $MgCl_2$, 2 mM dithiothreitol, and 5 mM Hepes, pH 7.3) and permeabilized for 5 min at 37°C with 1 IU/ml streptolysine O before addition of reporter and competitor peptides and ATP (2 mM). After incubation for 20 min at 37°C, cells were lysed by addition of 800 μl cold lysis buffer (500 mM NaCl, 50 mM Tris, 5 mM $MgCl_2$, and 1% Triton X-100, pH 7.4) with a cocktail of protease inhibitors. After a 30-min incubation on ice, lysates were clarified by centrifugation at 15,000 *g* for 10 min at 4°C, and supernatants were recovered for affinity purification of HLA-A2 molecules or glycosylated peptides.

Transport of Peptides with Glycosylation Acceptor Sequence. These assays were carried out as described by Neefjes et al. (31) using 1.5×10^6 cells in a volume of 100 μl and 160 nM iodinated reporter peptide. Reporter peptides R-10-T or S-11-R were used. Glycosylated reporter peptides were recovered by 2-h incubations of cleared supernatants with 100 μl Con A–Sepharose beads at 4°C. Beads were washed four times with assay buffer before counting of bound radioactivity. 100% accumulation corresponded to ~15,000 cpm.

Transport of Peptides Binding to HLA-A2. Based on published methods (32), 5×10^6 B cells/sample in 300 μl buffer with 130 nM reporter peptide were used. Reporter peptide S-9-L was used in TAP-proficient cells, and L-9-V was used in TAP-deficient cells. For TAP-independent peptide translocation, no ATP was added. After the incubation period, cells were pelleted to remove excess reporter peptide, washed in 800 μl buffer, and lysed. HLA-A2 molecules were then immunoprecipitated for 20 min (4°C) with 10 μg mAb BB7.2 preadsorbed on 15 μl protein A–Sepharose beads. 100% accumulation corresponded to ~15,000 cpm.

Peptides were iodinated by the chloramin T method using 12 nmol peptide, 0.5 mCi ^{125}I, and 0.5 mg/ml chloramin T (Merck) for peptides F-10-V, S-9-L, and L-9-V. For R-9-L, R-10-T, and S-11-R peptides, 1 mCi ^{125}I and 2 mg/ml chloramin T were used. Free iodine was removed on Sephadex G10 columns. For peptides S-9-L and L-9-V, 30% dimethylformamide was added to PBS labeling buffer. Specific activity of iodinated peptides was between 2.5 and 6×10^4 cpm/pmol.

In competition experiments, cpm corresponding to 50% inhibition of specific peptide binding to TAP or of accumulation of glycosylated or A2-bound peptide in the ER was determined graphically as described (8). Nonspecific binding, i.e., binding of iodinated peptide to control TAP1 microsomes, or peptide recovered upon incubation of Con A or BB7.2 beads with lysates of nonpermeabilized cells was <2% of specific binding in the absence of competitor unless indicated otherwise.

Purification of HLA-A2 Molecules

Purified mAb BB7.2 (or glycine at 10 mM) was coupled to cyanobromide-activated Sepharose 4B beads at a ratio of 7.5 mg mAb/ml resin. 3×10^9 Jesthom cells were lysed for 1 h at 4°C in 60 ml lysis buffer containing 50 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100, and a cocktail of protease inhibitors. Clarified lysate was precleared by incubation with 900 μl glycine–Sepharose beads for 3 h and incubated overnight at 4°C with 175 μl BB7.2–Sepharose (corresponding to 1.3 mg mAb). Beads were washed with a series of cold lysis buffers (5 ml each, with 0.1–0.5% Triton X-100) with pH 7.4, pH 8, and pH 9, and, finally, once with 1.8 ml of 250 mM NaCl, 0.1% Triton X-100, and 10 mM Tris, pH 9. HLA-A2 molecules were eluted twice with 500 μl of 50 mM triethanolamine, 150 mM NaCl, and 0.1% SDS, pH 11.5, and immediately neutralized by addition of 150 μl of 1 M Tris, pH 6.8. Eluates were dialyzed against PBS buffer containing 0.05% Triton X-100. HLA-A2 concentration in eluates was quantified in Coomassie-stained SDS-PAGE gels.

HLA-A2 Peptide Binding Assay

To measure peptide binding to HLA-A2, we developed an assay derived by modification of published procedures (33, 34). 350 ng of HLA-A2 was incubated in a total volume of 20 μl PBS with 0.05% Triton X-100 and protease inhibitors with 0.5 μg human β_2m, 3 μl iodinated reporter peptide F-10-V, and competitor peptides. After incubation for 2 d at 25°C, HLA-A2 molecules were separated from free peptide by size exclusion chromatography on Biogel P-30™ spun columns (Bio-Rad Labs.) equilibrated in PBS buffer containing 0.5% Triton X-100. Cpm eluting in the void volume corresponded to HLA-A2–bound peptide and was corrected by subtracting cpm leaking through columns loaded with peptide only. In the absence of competitor peptides, ~40,000 cpm reporter peptide bound to HLA-A2 molecules.

CTLs

Three approaches were used to generate CTL lines specific for HBV epitopes: human lines primed in vitro, lines from HBV patients, and lines from HLA-A2–transgenic mice. A total of nine independent CTL lines was used for experiments shown and/or referred to in this paper: for HBV pol 575-83, two lines from in vitro immunization, and one from a patient; for HBV core 18-27,

one line from in vitro immunization, two from patients, one from conventional and two from single chain–transgenic mice. As most CTL lines lost specificity upon freezing, it was not always possible to use the same line for subsequent experiments concerning one epitope; experiments were therefore generally repeated with different CTL lines.

In Vitro Induction of Peptide-specific CTL Lines. CTL lines were generated by coculture of PBMCs from healthy HLA-A2$^+$ donors with cells from a second donor with a high proliferative response to tetanus toxoid in a transwell system (Costar Corp.) using a modification of published procedures (35). In brief, PBMCs were seeded into 24-well plates at 4×10^6 cells/well in RPMI 1640 with 10% pooled human AB serum in the presence of 10 μg/ml HBV peptide, 1 μg/ml tetanus toxoid, and 10^6 PBMCs of the second donor in the inner well. Unlike in the published protocol, recombinant human IL-7 was added on day 3 at 50 U/ml. Cultures were restimulated weekly with autologous irradiated PBMCs prepulsed with peptide. Starting on day 7, cultures were maintained in 10 U/ml human IL-2 and 50 U/ml IL-7. Once CTL lines exhibited target cell lysis >30%, IL-2 concentration was increased to 50 U/ml. Almost 90% of peptide-specific CTL lines obtained by in vitro immunization did not recognize naturally processed peptide, a phenomenon already documented in previous reports (36).

Generation of HBV-specific CTL Lines from Patients with Acute HBV Infections. CTL lines from HBV-infected patients were generated exactly as described previously (37).

DNA Immunization of HLA-A2.1–transgenic Mice. D^b and mouse β_2m double-knockout mice expressing an HLA-A2.1/human β_2m (HHD) single chain molecule (30) and conventional HLA-A2–transgenic mice (38) were injected in the regenerating anterior tibialis muscle of each leg (5 d after injection of cardiotoxin in the same sites) with 50 μg cesium chloride–purified plasmids, corresponding to HBV sequences encoding the pol protein (nucleotides 2290–1874) or the core protein (1887–2800) cloned into pcDNA3™ (Invitrogen Corp.) with a CMV promoter. 3 wk later, spleens were removed and spleen cells were restimulated with an equal number of irradiated LPS blasts prepulsed with 1 μM peptide; peptide stimulation was repeated weekly until specific cytotoxic activity was detected. Blasts were generated by culture of spleen cells from HHD mice for 3 d at 10^6 cells/ml in RPMI supplemented with 25 μg/ml yeast LPS and 7 μg/ml dextran sulfate.

Vaccinia Viruses

Selected peptides were expressed as minigenes under the control of the p7.5 promoter in vector pSC11ss modified by insertion of a sequence containing BglII, KpnI, NsiI, and NotI sites between the SalI and StuI sites. Complementary oligonucleotides comprising an ATG initiation codon and a stop codon with appropriate cohesive ends were purchased from Genosys, annealed by slow cooling from 70 to 30°C, and ligated overnight at 14°C to modified pSC11ss. To express epitopes preceded by a signal sequence, oligonucleotides corresponding to the signal peptide of adenovirus E3/19K protein (20) were cloned into the KpnI/NotI sites of modified pSC11ss, followed by insertion of minigenes coding for epitopes HBV pol 575-83 or core 18-27 into the NotI/StuI sites downstream. Oligonucleotides corresponding to HBV pol 575-83 and 573-83 were cloned into the SalI/StuI sites; for peptides HBV core 18-27, 17-27, and 17-28, SalI/NsiI sites were used. Insertion of correct minigene sequences was verified by sequencing. Recombinant viruses were generated by cotransfection of CV-1 cells freshly infected by viral strain WR (ATCC) at a multiplicity of infection (MOI) of 0.1–1.0 with plasmids containing minigenes together with 150 ng wild-type vaccinia DNA, followed by selection of recombinant viral plaques in HuTK cells and large-scale production of viruses in HeLa S3 cells according to standard procedures. Recombinant vaccinia viruses coding for the entire HBV polymerase and core proteins have been described previously (37).

CTL Assay

Cytolytic activity was measured in 5-h ^{51}Cr-release assays using a standard protocol with 3,000 targets per well in RPMI 1640 supplemented with 5% FCS. When sensitization by recombinant vaccinia viruses was tested, 1.5×10^6 target cells were infected for 1.5 h at an MOI of 10 in medium with 2.5% FCS. Cells were washed once in 0.9% NaCl buffer, incubated for 3.5 h at 37°C in medium with 10% FCS, and then labeled with 100 μCi of $Na_2{}^{51}CrO_4$ for 1 h at 37°C. Cells were washed in 0.9% NaCl with 2% FCS, incubated for 1 h at 37°C, and washed again. To test sensitization by synthetic or HLA-A2–eluted peptides, target cells were incubated for 1.5 h during or after cell labeling with peptides. In experiments with brefeldin A (BFA), target cells were incubated for 2 h at 37°C with 5 μg/ml BFA; BFA concentration was maintained at 5 μg/ml throughout sensitization by peptide or virus by two-hourly replacement of medium. BFA concentration during the 5-h killing assay was 0.5 μg/ml (39). Percent specific lysis was calculated according to the formula, (experimental cpm − spontaneous release)/([total release/2] − spontaneous release); only experiments with <20% spontaneous release were considered.

Extraction and Analysis of Peptides Bound to HLA-A2 Molecules

Large-Scale Infection of Cells and Purification of HLA-A2 Molecules. HLA-A2–bound peptides were analyzed using a modification of published methods (21): 1.6 (or 1.3 in the experiment shown in Fig. 6, E and F) $\times 10^9$ JY cells were infected for 2 h at 10^8 cells/ml and an MOI of 10 (5 in Fig. 6, E and F) with vaccinia virus. After incubation for 6–8 h at 37°C, cells were harvested, washed once in cold PBS with 1 mM PMSF, and lysed as in the protocol for HLA-A2 purification. Clarified detergent lysate was precleared by incubation with 150 μl packed Sepharose beads coupled to mAb IVD12 (corresponding to 750 μg mAb) for 3 h and then incubated overnight at 4°C with 90 μl (i.e., 675 μg) BB7.2–Sepharose beads. IVD12 and BB7.2–Sepharose beads were washed with 10 bed volumes of 50 mM Tris, 150 mM NaCl, pH 8.0, with 0.5% NP-40 (Sigma Chemical Co.), then with 10 volumes of buffer without NP-40, and finally with 10 volumes of 10 mM Tris, pH 8.0. Material bound to beads was eluted by two 5-min incubations in three volumes of 0.1% TFA (Sigma Chemical Co.); eluted peptides were separated from higher molecular weight material by centrifugation through Centricon 10 (Amicon, Inc.) devices and finally dried by vacuum centrifugation.

HPLC Analysis and Fractionation. Dried eluate was resuspended in 100 μl 0.1% TFA and fractionated by reversed-phase HPLC using a SMART system (Pharmacia). μRPC C2/C18™ 2.1/10 columns were run in 0.1% TFA (solvent A) and 80% acetonitrile containing 0.081% TFA (solvent B). HPLC conditions were calibrated using synthetic peptides and chosen so as to allow maximal resolution of minimal epitopes and precursors. The following gradients were used: peptides HBV pol 575-83 and 573-83: 0–5 min, linear increase from 0% B to 48% B; 45–57 min, linear increase to 53% B; 57–65 min, linear increase to 90% B. Peptides HBV core 18-27 and 17-27: 0–40 min, linear increase from 0 to 38.8% B; 40–80 min, linear increase to 39.8% B; 80–90 min, linear increase to 90% B; a slightly different gradient was used in the ex-

periment in Fig. 6 E. Flow rate was maintained at 100 μl/min, and fractions of 100 μl (peptides pol 575-83 and 573-83) or 250 μl (peptides core 18-27 and 17-27, HBV core protein) were collected. Fractions were dried in a vacuum centrifuge and resuspended in 50 μl PBS with 5% DMSO. Fractionated peptide elutions were tested using TAP-deficient T2 or RMA-S HHD cells preincubated for 36 h at 27°C to increase surface expression of HLA-A2 (30) before pulsing with 5 μl of each fraction; in the experiment shown in Fig. 6 E, 20 μl of each fraction was used for pulsing.

Results

HLA-A2–presented Viral Epitopes Frequently Have Low TAP Affinities. To establish a system in which the biological effect of human TAP selectivity and potential ER processing of TAP-translocated peptides could be studied, we sought to identify peptides with low transporter affinities that might enter the ER exclusively in an extended form. Having previously observed that ligands for certain HCI molecules, especially HLA-A2, frequently display low affinities for human TAP (8, 14), we tested a panel of peptides with high or intermediate HLA-A2 binding affinity (6) (50% inhibiting concentration [IC_{50}] of 0.47–400 nM) derived from HBV and HCV proteins for binding to human TAP complexes (40). Among 34 tested peptides, 8 HBV peptides (Fig. 1) and 3 HCV peptides have been found to be antigenic in hepatitis patients and can therefore be naturally processed and presented to CTLs (29, 37). Relative affinities measurable in the TAP binding assay range from 0.1 to 3,000. 18/25 HBV peptides (Fig. 1), and 5/8 HCV peptides (not shown) derived from HBV proteins had low (IC_{50} >300) or unmeasurable TAP affinity. Thus, most peptides derived from HBV and HCV that bind to HLA-A2 and in many cases induce efficient CTL responses in vivo had low TAP affinities, raising the question of how these peptides obtain access to ER-resident HLA-A2 molecules for efficient presentation. Based on the criteria of strong antigenicity in vivo and low TAP affinity, we chose to study two HLA-A2–presented peptides with unmeasurable TAP affinity, HBV pol 575-83 and core 18-27.

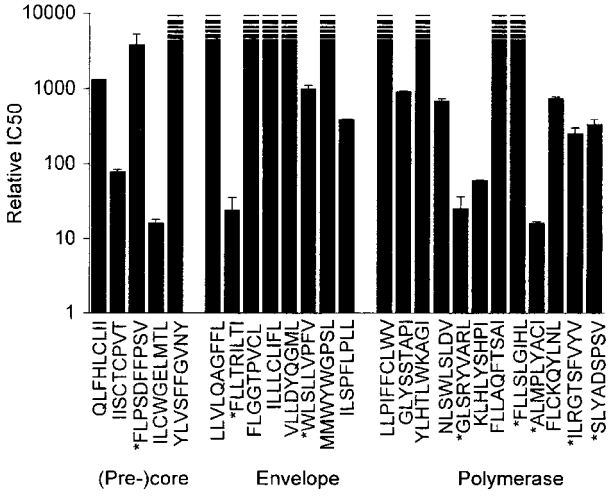

Figure 1. TAP binding affinity of HBV peptides binding to HLA-A2. Peptide sequences and HBV proteins of origin are indicated below the graph. Peptides marked with an asterisk are frequently recognized by CTLs from patients with acute HBV infections (37). Peptide affinities in this and all other panels are expressed as normalized IC_{50}, i.e., IC_{50} of competitor peptide divided by IC_{50} of unlabeled reporter peptide measured in the same assay. Columns with broken tops in this and other figures indicate unmeasurable competition efficiency, i.e., in this case IC_{50} values above 3,000.

HLA-A2 Binding Affinity of HBV Epitopes and Extended Variants. For each of the two HBV epitopes with very low TAP affinity, we designed precursor peptides by extending epitopes to the closest likely proteasome cleavage site (2, 24) flanking their NH_2 and/or COOH termini in the natural protein sequence. Sequences of minimal and extended peptides are shown in Fig. 2. Peptides extended by an Nt methionine residue were also tested, as such peptides may result from incomplete processing of minigene-expressed epitopes by cytosolic methionine aminopeptidase. We then proceeded to an in vitro evaluation of parameters that should affect efficiency of peptide presentation: HLA-A2 binding affinity, TAP binding and transport efficiency, and efficiency of TAP-independent ER access.

To measure HLA-A2 binding affinity, we adopted a published competition binding assay with immunoaffinity-purified HLA-A2 molecules (33, 34). Measurable competitor peptide affinities (IC_{50}) range from 0.1 to 100. Simple extension of epitope pol 575-83 by naturally found residues at either end surprisingly did not change HLA-A2 binding affinity (Fig. 2); however, Nt addition of methionine or extension at both ends decreased affinity. In contrast, all modifications of core 18-27 strongly reduced A2 affinity; this result is in accordance with a previous study that identified this peptide as an optimal synthetic epitope (41).

TAP Affinities of Epitopes and Extended Variants. TAP affinity, the second parameter likely to affect efficiency of presentation, was evaluated with the two available techniques,

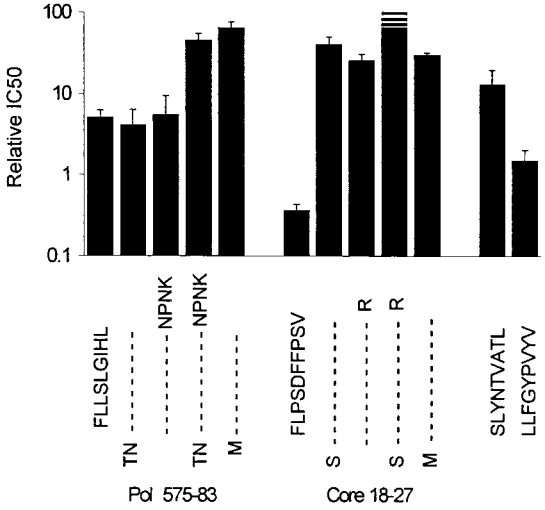

Figure 2. HLA-A2–binding affinities of selected epitopes and precursors. HLA-A2–binding affinities of epitopes and Nt- and/or Ct-extended variants were measured in competition binding assays using reporter peptide F-10-V; mean normalized IC_{50} values from three independent experiments are shown. The two sequences to the right correspond to reporter peptides S-9-L and L-9-V used in transport experiments shown in Fig. 4.

TAP binding and transport assays. Transport assays (Fig. 3 A) measure accumulation of glycosylated peptide in the ER of streptolysin O–permeabilized cells (31), which is TAP and ATP dependent but also affected by other processes, such as peptide degradation and active depletion from the ER. TAP binding assays (Fig. 3 B) evaluate at low temperature the initial step of peptide binding to the transporter in which substrate is selected (9, 40). Largely equivalent results were obtained with the two assays. For both epitopes, Nt extension by one or two residues increased TAP affinity to low to intermediate levels (IC_{50} of 57–372 in binding assay). Ct extension of the core epitope but not of pol 575-83 increased affinity, and combination of both extensions resulted in high TAP affinity of the core 17-28 variant (IC_{50} of 1.1). Ct-extended variants (pol 573-87 and core 18-28 peptides) competed less efficiently in transport than in binding assays; this may be due to peptide modification by cytosolic carboxypeptidases or peptide degradation in transport assays. Thus, for both epitopes, Nt-extended variants and, for the core epitope, also a Ct-extended variant with higher TAP affinity could be identified.

A Pathway with Low Efficiency Provides Access to the ER to Epitopes and Extended Variants in TAP-deficient Cells. Some peptides can be presented by HCI molecules in a TAP- and signal sequence–independent fashion (42). Moreover, some epitopes are presented with high efficiency in TAP-deficient T2 cells when expressed as minigenes (43). To test whether the selected epitopes and their variants could enter the ER in a TAP-independent manner, we adopted a transport assay in which labeled reporter peptides bind to newly synthesized HCI molecules and can be recovered by immunoprecipitation of the latter (32). To monitor TAP-independent peptide access to the ER, we used a human T cell lymphotrophic virus type 1–derived reporter peptide (L-9-V) with high binding affinity for HLA-A2 (Fig. 2) that has been shown to be presented efficiently in TAP-deficient cells when expressed as a minigene (43); assembly of L-9-V with HLA-A2 was measured in permeabilized T2 cells (Fig. 4 A).

The amount of L-9-V entering the ER and assembling with HLA-A2 decreased sevenfold in the absence of functional TAP complexes (not shown); addition of ATP did not increase recovery of HLA-A2–bound peptide, suggesting that TAP- and signal peptide–independent ER access is a relatively inefficient process that does not require ATP. As shown in Fig. 4 A, the two minimal epitopes and most single-extended precursor peptides competed for assembly of L-9-V with HLA-A2 in T2 cells and were therefore able

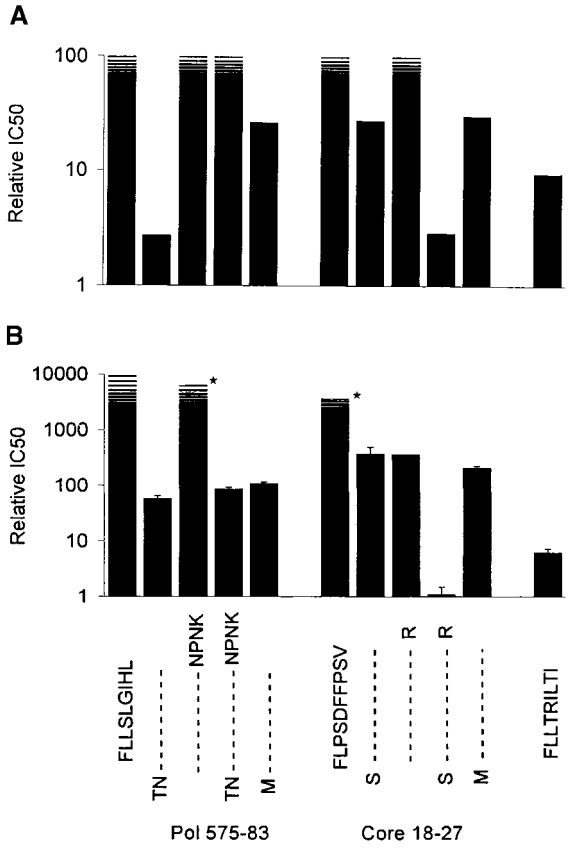

Figure 3. TAP affinities measured in transport and binding assays. (A) Inhibition by epitopes and their variants of accumulation of glycosylated reporter peptide R-10-T in streptolysin O–permeabilized cells was measured; results equivalent to those shown were obtained with a second reporter peptide, S-11-R. (B) Mean normalized IC_{50} values from three TAP binding experiments. Broken tops marked with an asterisk in Figs. 3 and 4 indicate values obtained by extrapolation.

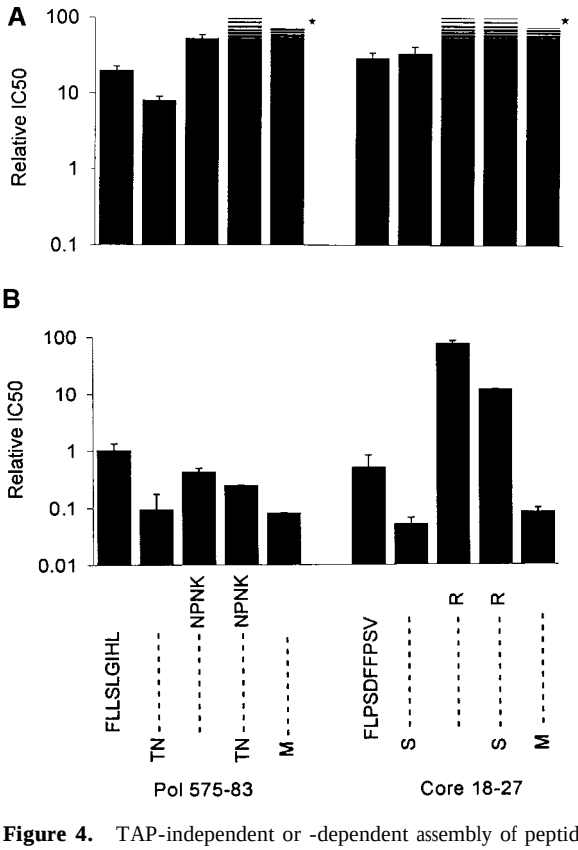

Figure 4. TAP-independent or -dependent assembly of peptides with HLA-A2 molecules in permeabilized cells. (A) Permeabilized HLA-$A2^+$ TAP-deficient T2 cells were incubated with labeled reporter peptide L-9-V; HLA-A2–bound reporter peptide was then quantified by immunoprecipitation with mAb BB7.2. (B) Permeabilized TAP-proficient $A2^+$ JY cells were incubated with reporter peptide S-9-L followed by immunoprecipitation. Unspecific reporter peptide assembly with HLA-A2 was ~10%.

to enter the ER in TAP-deficient cells; differences in IC_{50} values were relatively small. Double-extended precursors did not compete at all. High amounts of three unrelated 9-mer peptides binding to HLA-B40 and the HLA-B27–binding peptide S-9-R, a peptide reported to enter the ER when expressed as a minigene in TAP-deficient cells (43), also did not compete (not shown). This suggests that TAP-independent ER entry does not involve interaction with a saturable substrate binding site of a transporter or channel. As reporter and competitor peptides competed for TAP-independent ER access as well as binding to HLA-A2, it is possible that the observed differences were due to distinct HLA-A2 binding affinities. However, measured A2 affinities (Fig. 2) did not fully explain competition efficiencies in TAP-independent transport assays, so that distinct efficiencies of ER access are likely to contribute to experimental results. Thus, TAP-independent peptide entry into the ER of permeabilized cells displayed relatively low efficiency as well as selectivity and appeared not to correspond to an active process, as indicated by lack of ATP requirement and absence of sensitivity to competition.

Dominant Role of TAP Affinity for Efficiency of Assembly of Cytosolic Peptides with HLA-A2 In Vitro. We finally tested peptides in an in vitro assay that is likely to reflect the combined effect of the parameters evaluated above. In this assay, peptides competed for TAP-dependent and -independent ER entry and binding to HLA-A2 molecules of reporter peptide S-9-L with high TAP affinity (IC_{50} = 2.3; reference 8) and an intermediate HLA-A2 binding affinity (IC_{50} = 12.9; Fig. 2). The two epitopes showed a distinct pattern of relative competition efficiencies of minimal and extended epitope forms (Fig. 4 B). Isolated Nt extension by one or two residues (including a single methionine) significantly increased efficiency of competition; however, Ct or double extension slightly increased competition efficiency of the pol peptide, whereas it dramatically decreased that of the core peptide.

In view of the evaluation of isolated parameters described above, the distinct efficiencies of accumulation can be interpreted as follows. Increased efficiency of single Nt-extended variants could only be due to higher TAP affinities, as TAP-independent ER access of these peptides was unchanged and HLA-A2 affinities were equal (pol 573-83) or significantly decreased (methionine–pol 575-83, both core variants). Importantly, the low HLA-A2 affinities of three Nt-extended precursors did not appear to affect efficiency of competition; this suggests that either the negative effect on HLA-A2 affinity of Nt extension observed in equilibrium binding assays is not present during initial peptide assembly with HLA-A2 measured in the assay described here or that Nt extensions were removed by an ER-resident trimming activity during the assay, thereby increasing peptide affinity for HLA-A2.

Although Ct- or double-extended variants of HBV core 18-27 were very similar to Nt-extended variants with respect to reduced HLA-A2 affinities and increased TAP affinities, they competed for assembly with HLA-A2 in permeabilized cells with greatly reduced efficiency and thus differed strikingly from Nt-extended variants. In view of previous reports demonstrating the absence of a Ct trimming activity in the ER (10, 19), this discrepancy suggests that Nt but not Ct peptide trimming in the ER occurs during transport experiments as described here and that Ct-extended variants do not compete efficiently in these experiments because of their low affinity for HLA-A2. Alternatively, only the negative effect of Ct extensions on HLA-A2 affinity, but not that of Nt extensions observed in equilibrium binding assays, may act during initial peptide assembly with HLA-A2.

Taken together, HCI and TAP affinities both contributed to selection of epitope forms that can enter the ER and assemble with HCI molecules in our experimental system in vitro. Extended epitope forms with increased TAP affinities entered into the ER with greatly increased efficiency; subsequent efficient assembly with HLA-A2 was restricted to epitope forms with high HLA-A2 affinities and epitope precursors with lower HLA-A2 affinities due to Nt but not Ct extensions. However, a TAP-independent pathway provided access to the ER even to epitope forms with very low TAP affinities, albeit with reduced efficiency. As very small quantities of antigenic peptides can be sufficient for T cell recognition, and because some factors influencing peptide presentation by intact cells may not be readily measurable by the applied techniques in vitro, we decided to examine the importance of the described in vitro findings for presentation of the various epitope forms to specific CTL lines.

TAP Affinity Determines Efficiency of Presentation of Peptides Expressed as Minigenes. We expressed minimal epitopes, Nt-extended precursors, and minimal epitopes preceded by the E3/19K signal sequence as minigenes in recombinant vaccinia viruses in order to obtain high peptide levels in the cytosol or ER, respectively. In parallel, HLA-A2–restricted specific CTL lines were generated using three different approaches: in vitro priming and expansion of PBMCs from healthy donors by successive restimulation with autologous irradiated PBMCs pulsed with synthetic minimal epitopes (35), short-term expansion of secondary CTL lines by peptide restimulation of PBMCs from patients with acute HBV infections (37), DNA immunization with plasmids encoding full-length HBV proteins of two transgenic mouse lines expressing HLA-A2/K^b heavy chain and human β_2m encoded by two transgenes (38), or a single chain construct (30). All used CTL lines met the following criteria: (a) specific recognition of target cells pulsed with synthetic minimal and Nt-extended epitope variants and (b) recognition of naturally processed epitope presented by HLA-$A2^+$ target cells.

Most CTL lines were tested for relative efficiencies of recognition of short and extended epitope forms. All tested lines specific for pol 575-83 and core 18-27 recognized both short and Nt-extended epitope forms; however, recognition of epitope precursors generally required 10–50-fold higher peptide concentrations than that of minimal epitopes (not shown). In the case of the core epitope, CTL recognition of Nt-extended longer peptides is also in accordance with a previous study (41). CTL recognition of

synthetic precursor peptides was not due to contamination by truncated peptides (44); high resolution HPLC analysis of synthetic precursor peptides combined with mass spectrometric analysis demonstrated absence of contaminating minimal epitopes (not shown). CTL lines generated by immunization in vivo (patients/transgenic mice) generally lost recognition of short synthetic peptides at 10^{-12} M, whereas lines derived by in vitro immunization lost recognition at 10^{-10} M. To ascertain that experiments with vaccinia viruses reflected intracellular assembly of peptides with HLA-A2 molecules and not binding to cell surface HLA-A2 molecules of peptides released from lysed infected cells, we also performed control experiments in which export of newly ER-assembled HLA-A2 molecules to the cell surface was inhibited by BFA (39). As expected, this treatment completely inhibited presentation of minigene-expressed epitopes but did not affect presentation of synthetic peptides (not shown).

As shown in Fig. 5, CTL lines recognizing epitope pol 575-83 killed target cells much more efficiently when these expressed the Nt-extended precursor 573-85 than cells expressing the minimal peptide 575-83. Lysis of cells expressing the precursor was similar to lysis of cells pulsed with short synthetic peptides or expressing full-length pol protein, whereas cells expressing the minimal epitope were hardly recognized at all in an experiment with a CTL line from a patient (Fig. 5 A). A similar although less dramatic difference in recognition was obtained with two CTL lines obtained by in vitro immunization (Fig. 5 B and data not shown). In contrast, one of these lines recognized TAP-deficient T2 cells expressing the two epitope forms with equal, intermediate efficiency, demonstrating that more efficient sensitization by expression of the precursor form was due to TAP-mediated ER access of this epitope variant (Fig. 5 C).

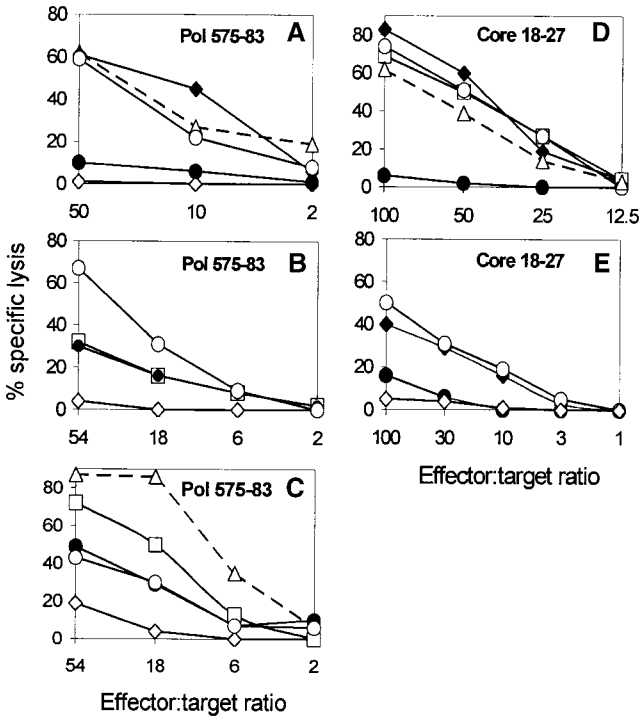

Figure 5. Efficiency of presentation of minigene-expressed minimal epitopes and Nt-extended precursors to specific CTL lines. Target cells were sensitized by synthetic peptides, by vaccinia viruses corresponding to different forms of epitopes indicated in the panels, or by control viruses, and lysis by CTL lines specific for the indicated epitopes was measured. Symbols indicate target cell sensitization by the following reagents: △, synthetic peptide corresponding to minimal epitope; ◇, irrelevant vaccinia virus; ◆, vaccinia virus expressing the appropriate full-length HBV protein; □, vaccinia-expressed short epitope preceded by the E3/19K signal sequence; ●, vaccinia-expressed short epitope; ○, vaccinia-expressed, Nt-extended precursor peptide. (A) Lysis of JY target cells by a patient-derived CTL line. (B) Lysis of Jesthom cells by a line derived by in vitro immunization. (C) Lysis of T2 cells by the same line as in B. (D and E) Lysis of JY cells by two CTL lines derived from different acutely HBV-infected patients.

An even more dramatic effect of Nt epitope extension was observed for epitope core 18-27 and its precursor, 17-27 (Fig. 5 D). In this case, the HBV patient–derived line did not kill cells expressing the minimal epitope but recognized precursor peptide–expressing cells and cells expressing the core protein or the signal peptide–coupled epitope with equally high efficiency. Equivalent results were obtained with a CTL line derived from another patient (Fig. 5 E) and a line generated by in vitro immunization (not shown).

In conclusion, investigation of presentation of minigene-expressed epitopes and Nt precursors confirmed results obtained in vitro and underlined the dominant role of TAP in selecting variants of two HLA-A2–presented epitopes for presentation, as well as the low efficiency of TAP-independent ER access. Concordant results of in vitro and ex vivo experiments suggested that all major parameters controlling presentation of the two epitopes had been analyzed in vitro. However, it was not clear whether the TAP-selected precursors were presented in an unmodified or shortened form.

A TAP-translocated Precursor Peptide with Low HLA-A2 Binding Affinity Undergoes Nt Processing in the ER. To find out whether precursor peptides could be processed in the ER, we analyzed HLA-A2–presented peptides from minigene-expressing cells. HLA-A2 molecules were purified from JY cells expressing minimal or Nt-extended forms of epitopes pol 575-83 and core 18-27, and peptides associated with purified HLA-A2 molecules were analyzed according to published methods (21). Acid-eluted peptides were fractionated under HPLC conditions that allowed maximal separation of synthetic peptides corresponding to minimal epitopes and the two Nt-extended precursors for each of the epitopes; peptides extended by an Nt methionine were included in calibration experiments to allow detection of vaccinia-expressed minimal epitopes having undergone incomplete processing by cytosolic methionine aminopeptidase. Each fraction was tested for its capacity to sensitize HLA-$A2^+$ cells for lysis by specific CTL lines. Unspecific lysis was determined as the mean lysis obtained by sensitization with fractions from control IVD12-coated beads and was always $<15\%$.

In the case of epitope pol 575-83, only the epitope forms encoded by the minigenes could be detected in fractions eluted from HLA-A2 (Fig. 6 A). Specifically, elutions from cells expressing precursor 573-85 did not contain sensitizing activity with the retention time corresponding to the minimal epitope; in addition, elutions from cells expressing

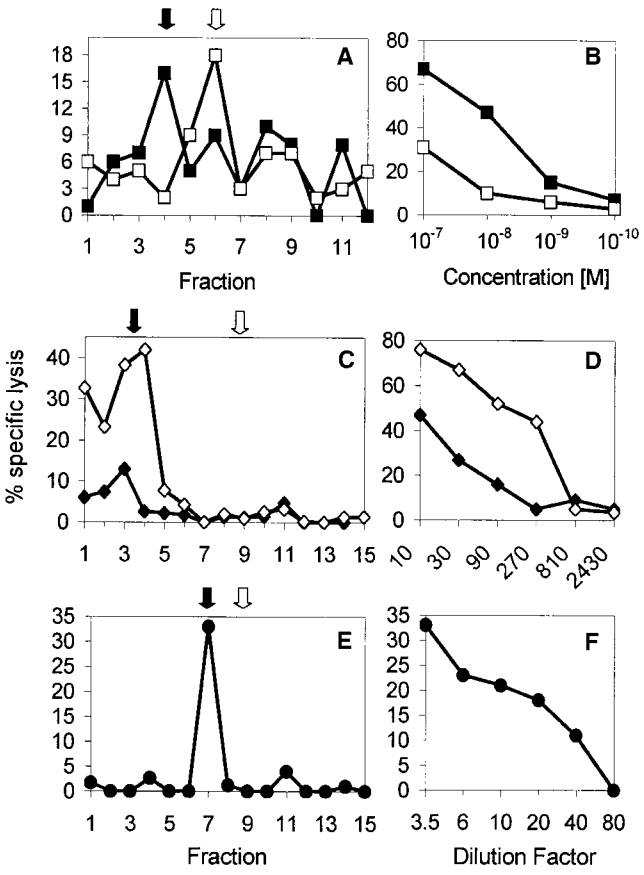

Figure 6. Peptides presented by cells expressing minimal or Nt-extended epitopes. HLA-A2 molecules were purified from JY cells expressing vaccinia virus–encoded minigenes, and peptides eluted by acid treatment of A2 molecules were fractionated and tested for recognition by specific CTL lines. Elution times of synthetic peptides analyzed under identical HPLC conditions are indicated in A, C, and E by solid arrows (minimal epitope forms) and open arrows (Nt-extended precursors), respectively. In A–D, filled symbols represent elutions from cells expressing minimal epitopes (or incubation with the corresponding synthetic peptide in B), and open symbols correspond to Nt-extended precursors. Target cells were T2 cells in A–C and RMA-S HHD transfectants in D–F. CTL lines were derived by in vitro immunization (A and B, same line used as in Fig. 5, B and C), from an HBV patient (C), or from two different HHD-transgenic mice (one line used in D, the other used in E and F). (A) Sensitization by fractionated peptides from cells expressing HBV pol 575-83 or its Nt-extended precursor 573-83. (B) Efficiency of CTL recognition of synthetic peptides HBV pol 575-83 and pol 573-83. (C) Sensitization by A2-eluted peptide fractions from cells expressing HBV core 18-27 or its precursor, core 17-27. (D) Sensitization by dilutions of fraction 3 eluted from HLA-A2 molecules of cells expressing HBV core 18-27 or core 17-27. (E) Sensitization by fractionated peptides from cells expressing vaccinia-encoded HBV core protein. (F) Sensitization by dilutions of fraction 7 eluted from HLA-A2 molecules of cells expressing vaccinia-encoded HBV core protein.

the short epitope form did not contain sensitizing material in the fraction corresponding to the methionine-extended epitope (not shown). The same result was obtained with a second CTL line obtained from an HLA-A2– transgenic HHD mouse (not shown). Thus, there was no evidence for cleavage of the pol 573-83 precursor peptide in the ER.

Significant amounts of the short epitope were detected in HLA-A2–eluted material from cells expressing the minimal epitope pol 575-83 (Fig. 6 A). We have so far not been able to quantify the peptide contained in these fractions, although indirect evidence allows us to conclude that elutions from precursor peptide–expressing cells are likely to contain significantly more sensitizing peptide than those from cells expressing the short epitope. Due to its generation by in vitro immunization with pol 575-83, the CTL used for analysis of fractions recognized peptide 575-83 with 40–50-fold higher efficiency than peptide 573-83 (Fig. 6 B). As CTL recognition of sensitizing fractions from cells expressing pol 575-83 and 573-83 was equally efficient, it is likely that fractions from cells expressing and presenting peptide 573-83 contain substantially more peptide than those from cells expressing peptide 575-83. Detection of a low amount of peptide 575-83 in elutions from pol 575-83/vaccinia–infected cells is consistent with low-level killing of such cells by the same CTL line (Fig. 5 B).

Results for elutions from cells expressing the core epitope or its precursor were strikingly different from those for the pol epitope (Fig. 6, C and D). Both cells synthesizing the short and the extended epitope form exclusively presented the minimal epitope; in addition, HLA-A2 molecules from cells expressing the precursor peptide contained substantially larger quantities of this peptide than A2 molecules from cells expressing the short form (Fig. 6 C). The epitope variant extended by an Nt methionine could not be detected in cells expressing core 18-27 preceded by a methionine initiation codon (not shown). Results shown in Fig. 6 C were obtained with a patient-derived CTL line; equivalent results were obtained with three other CTL lines raised in conventional or HHD-transgenic mice (not shown). Thus, in cells expressing precursor peptide HBV core 17-27, apparently complete Nt processing in the ER was observed. When peptides eluted from cells expressing complete HBV core protein were analyzed, sensitizing activity also comigrated exclusively with the fraction corresponding to peptide core 18-27 (Fig. 6, E and F), demonstrating that this peptide is also the dominant epitope form generated from the source protein.

Detection of peptide HBV core 18-27 but not 17-27 might be due to more efficient CTL recognition of the shorter peptide, a standard feature of all CTL lines described in this paper, including the two lines generated from HHD-transgenic mice used for analysis of eluted material (Fig. 6, C and E). To address this issue, dilutions of synthetic peptides and of sensitizing fractions of eluted material were analyzed for CTL recognition. Both CTL lines recognized peptide 18-27 with 10-fold higher efficiency than 17-27 (not shown). CTL lines recognized a 500-fold dilution of the fraction from cells expressing the core 17-27 minigene (Fig. 6 D) and a 40-fold dilution of the fraction from cells expressing the HBV core protein (Fig. 6 F). Consequently, cells expressing minigene core 17-27 contained at least 50 times more peptide core 18-27 than 17-27, whereas cells expressing HBV core protein contained at least four times more of the short peptide. Titration experiments with synthetic peptide standards and elution fractions also allowed us to calculate the number of HLA-A2–extracted HBV core

18-27 molecules per cell. This number was 3,610 for cells expressing the core 17-27 minigene, 438 for the core 18-27 minigene, and 20 for core protein.

Discussion

Two principal novel findings emerge from this study: (a) the selectivity of human TAP can have a substantial biological impact on peptide entry into the ER and (b) similar to signal peptide or Jaw 1–coupled epitopes, TAP-transported peptides can be modified by a highly efficient aminopeptidase activity in the ER. To our knowledge, this is the first conclusive demonstration that the human peptide transporter can participate in peptide selection for presentation by HCI molecules. Thus, previously established rules governing peptide affinity for human TAP (8, 14) are biologically relevant.

In a comparison of the data reported here with earlier demonstrations of the biological impact of murine and rat TAP-B transporters (10, 12), an important difference needs to be emphasized. Significant biological effects of peptide selection by restrictive rodent transporters have been demonstrated in settings where TAP and MHC preferences for Ct peptide residues were incompatible. Due to the absence of Ct peptide processing in the ER (10), these incompatibilities cannot be compensated for after TAP transport and impair the efficiency of class I antigen processing so severely that incompatible TAP/MHC combinations have not survived in rat evolution (11). This study addresses the biological impact of TAP selection in a setting where Ct preferences of TAP and MHC class I molecules are adapted overall (as is the case in humans), and some peptides display low TAP affinities due to Nt as well as Ct sequence elements. In this setting, we find a dramatic effect of TAP affinity similar to previous studies of rodent transporters. However, in the absence of an overwhelming conflict between Ct preferences of human TAP and MHC class I molecules, transport of Nt-extended peptides followed (when required) by Nt processing in the ER allows highly efficient presentation of epitopes with very low TAP affinities. This suggests that, in humans, high permissiveness of the class I processing system downstream from proteolytic peptide generation is not due to absence of selectivity of TAP transporters but to a combination of TAP transport of epitope variants with Nt peptide processing in the ER.

Our analysis of a panel of HBV- and HCV-derived peptides (Fig. 1), with many of them representing in vivo immunodominant epitopes (37), suggests that precursor peptide selection by human TAP is a relatively common occurrence for HLA-A2–presented peptides. The frequent requirement for ER processing of ligand precursors for this molecule is likely to reflect the poor compatibility of HLA-A2 and TAP preferences. In a recent analysis of the compatibility of human TAP preferences with those of nine HCI molecules, HLA-A2 ligands showed the poorest and HLA-B27 ligands the best adaptation to TAP selectivity (14). However, close analysis of the TAP affinities of in vivo antigenic HBV peptides (Fig. 1) suggests that antigen processing may favor peptides that do not require ER processing. Whereas only 2/12 peptides with very low TAP affinities ($IC_{50} > 1{,}000$) are known to be antigenic in vivo, this is the case for 3/6 peptides with intermediate to high TAP affinities ($IC_{50} < 100$). This may indicate limited efficiency of the ER aminopeptidase or a requirement of specific flanking sequences for its activity.

As it is not possible to quantitate antigenic peptides in the cytosol, we do not know the expression levels of the minigene-expressed epitope forms. In a study by Anton et al., vaccinia virus–expressed, MHC class I–bound epitopes were detected at at least 50,000 copies per cell (45). We cannot entirely rule out that 9- or 10-mer epitope forms are produced at lower levels than 11-mer precursors, although there is no experimental evidence supporting this hypothesis. However, in complete accordance with results obtained with minigene-infected cells, synthetic precursor peptides assembled much more efficiently with HLA-A2 in permeabilized cells than shorter minimal epitopes. This suggests strongly that the dramatic difference between the efficiencies of sensitizations by minigene-expressed precursor and minimal peptides was due to more efficient TAP transport of precursors and not to lower expression of shorter epitope forms. We therefore conclude that, in the studied cases, high cytosolic concentrations of minimal epitopes could not compensate completely for low TAP affinities. It is likely that ER entry of minimal or extended epitope forms produced at physiological, i.e., lower concentrations generally requires higher TAP affinities than in the case of the two HBV-derived epitopes and their variants.

For the core 18-27 epitope, we were able to determine the nature and number of peptide molecules eluted from HLA-A2 molecules of cells expressing different epitope forms. This analysis revealed that peptide core 18-27 is the dominant HLA-A2–bound epitope form in cells expressing its precursor 17-27 and also in cells expressing the complete HBV core protein. Thus, in the case of this immunodominant HBV epitope, the naturally processed epitope form is excluded from the ER by low TAP affinity, demonstrating that TAP-imposed ER import of epitope precursors for luminal processing plays a role in natural antigen processing. Whatever the nature of precursor peptides of core 18-27 generated by cytosolic degradation of the HBV core protein, such precursor peptides must undergo ER processing similar to peptide core 17-27. Quantitative analysis of eluted fractions also demonstrated almost 10-fold higher amounts of core 18-27 peptide in elutions from cells expressing the precursor peptide, as compared with cells expressing the minimal peptide. The relatively low number of 20 peptide molecules per cell expressing HBV core is compatible with immunodominance of this epitope in HBV-infected patients. As few as three peptide molecules per cell can give rise to efficient CTL lysis (46). Anton et al. found a low number of 30 molecules per cell for an immunodominant viral epitope generated from the vaccinia-encoded source protein; as in this study, peptide copy number was dramatically increased upon expression of the epitope as minigene (45). Results of Pamer et al. also suggest that

immunodominance does not require high peptide copy numbers (47).

The relatively high peptide copy number in cells expressing the core 18-27 minigene clearly conflicts with CTL recognition of vaccinia-infected targets (Fig. 5); three independent CTL lines from different individuals recognized targets expressing whole core protein with high efficiency but targets expressing core 18-27 barely or not at all. We propose that the number of 438 peptide molecules per cell may be due to binding of cytosolic peptides to HLA-A2 molecules during overnight incubation of cell lysates for A2 immunoprecipitation. Although current consensus holds that antigenic peptides can only be recovered from cells expressing a restricting MHC class I molecule (1, 48), to our knowledge there is no experimental evidence to rule out that peptide–MHC complexes can be formed after cell lysis. Based on reports that as many as 50,000 peptides per cell can be presented by MHC class I molecules in cells expressing vaccinia-encoded minigenes (45), it can conservatively be assumed that at least 100,000 peptide molecules per cell are synthesized in such cells. Provided that these peptides are not (or not entirely) degraded and at least partly set free in detergent lysates, this would correspond to a maximum peptide concentration of 8 nM during immunoprecipitation of HLA-A2 under the conditions employed in this study. This concentration is not very far from the K_D of 40 nM of peptide core 18-27 (Lauvau, G., and P.M. van Endert, unpublished data). Corroboration of the hypothesis of cytosolic peptide association with HLA-A2 after cell lysis will require lysate mixing experiments followed by peptide elution and analysis.

Based on the identification of epitopes that cannot enter the ER in their minimal form, we have been able to study ER processing of TAP-translocated peptides. We find that precursor peptide pol 573-83 is presented without modification, presumably due to its high-affinity binding to HLA-A2; efficient binding of up to 12-mer peptides to HLA-A2 has been reported previously (49). Apparently complete processing of precursor peptide core 17-27 with low HLA-A2 affinity suggests that ER processing of precursor peptides may be controlled by HCI binding affinity but unaffected by peptide length; both precursor peptides are 11-mers.

Although the reported data establish clearly that TAP-transported precursor peptides can be processed in the ER, it remains unclear whether the involved mechanism is identical to that implicated in release of epitopes coupled to signal sequences (19). We have so far not been able to investigate in our system the effect of protease inhibitors that have been reported to inhibit assembly of signal sequence–derived peptides with HLA-A2 (50). Although the observation of an unusual length of signal sequence–derived peptides in TAP-deficient cells (27) may indicate distinct peptide maturation mechanisms in the two cases, involvement of an identical or similar mechanism is suggested by the apparent lack of Ct processing in both systems (19, 21). It is also possible that several proteolytic events are involved in epitope generation from signal sequences, only one of which also acts on peptides delivered by TAP.

We can now only speculate on the mechanism of Nt peptide processing. Our analysis of HLA-A2–eluted peptides may provide clues in this regard. Nt peptide processing may be carried out by a fluid phase or directly HCI-associated aminopeptidase. Although both scenarios could explain processing of precursor core 17-27, the complete absence of processing of precursor pol 573-83, as detected in elutions, is difficult to reconcile with random fluid phase processing. We therefore favor the alternative scenario, in which unstable HCI association of Nt-extended epitope precursors would be detected by a sensor mechanism integrated into or coupled to the putative peptidase. Sensor and processing mechanisms may preferentially associate with HCI molecules interacting with TAP–tapasin complexes. This hypothesis could also account for the frequent finding of long peptides bound to HLA-A2 in TAP-deficient cells and to HLA-B27 in normal cells; a recent report suggests that HLA-B27 may frequently assemble with peptides in a tapasin-independent fashion (26). Several proteins involved in peptide loading on HCI molecules are candidates for the putative role of aminopeptidase. These include HCI molecules themselves (51), the ER-resident chaperone gp96 (52), and the putative chaperone tapasin (4).

We gratefully acknowledge technical help by H. Cohen and P. Fowler throughout this study.

This work was supported by institutional grants from INSERM to P.M. van Endert and by National Institutes of Health grants RO1-AI20001 and RO1-CA40489 to F.V. Chisari.

Address correspondence to Peter van Endert, INSERM U25, 161 rue de Sèvres, 75743 Paris cedex 15, France. Phone: 33-1-44492563; Fax: 33-1-43062388; E-mail: vanendert@necker.fr

Submitted: 30 October 1998 Revised: 26 July 1999 Accepted: 26 August 1999

References

1. Pamer, E., and P. Cresswell. 1998. Mechanisms of MHC class I–restricted antigen processing. *Annu. Rev. Immunol.* 16: 323–358.
2. Tanaka, K., N. Tanahashi, C. Tsurumi, K.Y. Yokota, and N. Shimbara. 1997. Proteasomes and antigen processing. *Adv. Immunol.* 64:1–38.
3. Hansen, T.H., and D.R. Lee. 1997. Mechanism of class I assembly with beta 2 microglobulin and loading with peptide.

Adv. Immunol. 64:105–137.
4. Ortmann, B., J. Copeman, P.J. Lehner, B. Sadasivan, J.A. Herberg, A.G. Grandea, S.R. Riddell, R. Tampe, T. Spies, J. Trowsdale, et al. 1997. A critical role for tapasin in the assembly and function of multimeric MHC class I-TAP complexes. *Science.* 277:1306–1309.
5. Deng, Y., J.W. Yewdell, L.C. Eisenlohr, and J.R. Bennink. 1997. MHC affinity, peptide liberation, T cell repertoire, and immunodominance all contribute to the paucity of MHC class I-restricted peptides recognized by antiviral CTL. *J. Immunol.* 158:1507–1515.
6. Sette, A., A. Vitiello, B. Reherman, P. Fowler, R. Nayersina, W.M. Kast, C.J. Melief, C. Oseroff, L. Yuan, J. Ruppert, et al. 1994. The relationship between class I binding affinity and immunogenicity of potential cytotoxic T cell epitopes. *J. Immunol.* 153:5586–5592.
7. Momburg, F., J.J. Neefjes, and G. Hämmerling. 1994. Peptide selection by MHC-encoded TAP transporters. *Curr. Opin. Immunol.* 6:32–37.
8. van Endert, P.M., D. Riganelli, G. Greco, K. Fleischhauer, J. Sidney, A. Sette, and J.-F. Bach. 1995. The peptide-binding motif for the human transporter associated with antigen processing. *J. Exp. Med.* 182:1883–1895.
9. Gubler, B., S. Daniel, E.A. Armandola, S. Caillat-Zucman, and P.M. van Endert. 1998. Substrate selection by transporters associated with antigen processing occurs during peptide binding to TAP. *Mol. Immunol.* 35:427–433.
10. Powis, S.J., L.L. Young, E. Joly, P.J. Barker, L. Richardson, R.P. Brandt, C.J. Melief, J.C. Howard, and G.W. Butcher. 1996. The rat *cim* effect: TAP allele-dependent changes in a class I MHC anchor motif and evidence against C-terminal trimming of peptides in the ER. *Immunity.* 4:159–166.
11. Joly, A.L., A.L. Le Rolle, A.L. Gonzalez, W.J. Mehling, W.J. Stevens, W.J. Coadwell, J.C. Hunig, J.C. Howard, and G.W. Butcher. 1998. Co-evolution of rat TAP transporters and MHC class I RT1-A molecules. *Curr. Biol.* 8:169–172.
12. Yellen-Shaw, A.J., C.E. Laughlin, R.M. Metrione, and L.C. Eisenlohr. 1997. Murine transporter associated with antigen presentation (TAP) preferences influence class I–restricted T cell responses. *J. Exp. Med.* 186:1655–1662.
13. Momburg, F., J. Roelse, J.C. Howard, G.W. Butcher, G.J. Hämmerling, and J.J. Neefjes. 1994. Selectivity of MHC-encoded peptide transporters from human, mouse and rat. *Nature.* 367:648–651.
14. Daniel, S., V. Brusic, S. Caillat-Zucman, N. Petrovsky, L. Harrison, D. Riganelli, F. Sinigaglia, F. Gallazzi, J. Hammer, and P.M. van Endert. 1998. Relationship between peptide selectivities of human transporters associated with antigen processing and HLA class I molecules. *J. Immunol.* 161:617–624.
15. Neisig, A., J. Roelse, A.J. Sijts, F. Ossendorp, M.C. Feltkamp, W.M. Kast, C.J. Melief, and J.J. Neefjes. 1995. Major differences in transporter associated with antigen presentation (TAP)-dependent translocation of MHC class I-presentable peptides and the effect of flanking sequences. *J. Immunol.* 154:1273–1279.
16. Vijh, S., I.M. Pilip, and E.G. Pamer. 1998. Effect of antigen-processing efficiency on in vivo T cell response magnitudes. *J. Immunol.* 160:3971–3977.
17. Androlewicz, M.J., and P. Cresswell. 1996. How selective is the transporter associated with antigen processing? *Immunity.* 5:1–5.
18. Yewdell, J.W., H.L. Snyder, I. Bacik, L.C. Anton, Y. Deng, T.W. Behrens, T. Bachi, and J.R. Bennink. 1998. TAP-independent delivery of antigenic peptides to the endoplasmic reticulum: therapeutic potential and insights into TAP-dependent antigen processing. *J. Immunother.* 21:127–131.
19. Snyder, H.L., I. Bacik, J.W. Yewdell, T.W. Behrens, and J.R. Bennink. 1998. Promiscuous liberation of MHC-class I-binding peptides from the C termini of membrane and soluble proteins in the secretory pathway. *Eur. J. Immunol.* 28:1339–1346.
20. Snyder, H.L., J.W. Yewdell, and J.R. Bennink. 1994. Trimming of antigenic peptides in an early secretory compartment. *J. Exp. Med.* 180:2389–2394.
21. Elliott, T., A. Willis, V. Cerundolo, and A. Townsend. 1995. Processing of major histocompatibility class I–restricted antigens in the endoplasmic reticulum. *J. Exp. Med.* 181:1481–1491.
22. Snyder, H.L., I. Bacik, J.R. Bennink, G. Kearns, T.W. Behrens, T. Bachi, M. Orlowski, and J.W. Yewdell. 1997. Two novel routes of transporter associated with antigen processing (TAP)-independent major histocompatibility complex class I antigen processing. *J. Exp. Med.* 186:1087–1098.
23. Koopmann, J.O., M. Post, J.J. Neefjes, G.J. Hämmerling, and F. Momburg. 1996. Translocation of long peptides by transporters associated with antigen processing (TAP). *Eur. J. Immunol.* 26:1720–1728.
24. Niedermann, G., G. King, S. Butz, U. Birsner, R. Grimm, J. Shabanowitz, D.F. Hunt, and K. Eichmann. 1996. The proteolytic fragments generated by vertebrate proteasomes: structural relationships to major histocompatibility complex class I binding peptides. *Proc. Natl. Acad. Sci. USA.* 93:8572–8577.
25. Urban, R.G., R.M. Chicz, W.S. Lane, J.L. Strominger, A. Rehm, M.J. Kenter, F.G. Uytdehaag, H. Ploegh, B. Uchanska-Ziegler, and A. Ziegler. 1994. A subset of HLA-B27 molecules contains peptides much longer than nonamers. *Proc. Natl. Acad. Sci. USA.* 91:1534–1538.
26. Peh, C.A., S.R. Burrows, M. Barnden, R. Khanna, P. Cresswell, D.J. Moss, and J. McCluskey. 1998. HLA-B27-restricted antigen presentation in the absence of tapasin reveals polymorphism in mechanisms of HLA class I peptide loading. *Immunity.* 8:531–542.
27. Wei, M.L., and P. Cresswell. 1992. HLA-A2 molecules in an antigen-processing mutant cell contain signal sequence-derived peptides. *Nature.* 356:443–446.
28. Nayersina, R., P. Fowler, S. Guilhot, G. Missale, A. Cerny, H.J. Schlicht, A. Vitiello, R. Chesnut, J.L. Person, A.G. Redeker, et al. 1993. HLA A2 restricted cytotoxic T lymphocyte responses to multiple hepatitis B surface antigen epitopes during hepatitis B virus infection. *J. Immunol.* 150:4659–4671.
29. Cerny, A., J.G. McHutchison, C. Pasquinelli, M.E. Brown, M.A. Brothers, B. Grabscheid, P. Fowler, M. Houghton, and F.V. Chisari. 1995. Cytotoxic T lymphocyte response to hepatitis C virus–derived peptides containing the HLA A2.1 binding motif. *J. Clin. Invest.* 95:521–530.
30. Pascolo, S., N. Bervas, J.M. Ure, A.G. Smith, F.A. Lemonnier, and B. Perarnau. 1997. HLA-A2.1-restricted education and cytolytic activity of $CD8^+$ T lymphocytes from β2 microglobulin (β2m) HLA-A2.1 monochain transgenic H-2Db β2m double knockout mice. *J. Exp. Med.* 185:2043–2051.
31. Neefjes, J.J., F. Momburg, and G.J. Hammerling. 1993. Selective and ATP-dependent translocation of peptides by the MHC-encoded transporter. *Science.* 261:769–771.
32. Androlewicz, M.J., K.S. Anderson, and P. Cresswell. 1993. Evidence that transporters associated with antigen processing translocate a major histocompatibility complex class I-bind-

ing peptide into the endoplasmic reticulum in an ATP-dependent manner. *Proc. Natl. Acad. Sci. USA*. 90:9130–9134.
33. Olsen, A.C., L.O. Pedersen, A.S. Hansen, M.H. Nissen, M. Olsen, P.R. Hansen, A. Holm, and S. Buus. 1994. A quantitative assay to measure the interaction between immunogenic peptides and purified class I major histocompatibility complex molecules. *Eur. J. Immunol.* 24:385–392.
34. Sette, A., J. Sidney, M.F. del Guercio, S. Southwood, J. Ruppert, C. Dahlberg, H.M. Grey, and R.T. Kubo. 1994. Peptide binding to the most frequent HLA-A class I alleles measured by quantitative molecular binding assays. *Mol. Immunol.* 31:813–822.
35. Cerny, A., P. Fowler, M.A. Brothers, M. Houghton, H.J. Schlicht, and F.V. Chisari. 1995. Induction in vitro of a primary human antiviral cytotoxic T cell response. *Eur. J. Immunol.* 25:627–630.
36. Tanzarella, S., K. Fleischhauer, P. van Endert, C. Bordignon, and C. Traversari. 1997. Characterization of antigenic peptide epitopes by reverse immunology: induction of cytotoxic T lymphocytes specific for exogenous peptide only. *Int. J. Cancer*. 72:912–915.
37. Rehermann, B., P. Fowler, J. Sidney, J. Person, A. Redeker, M. Brown, B. Moss, A. Sette, and F.V. Chisari. 1995. The cytotoxic T lymphocyte response to multiple hepatitis B virus polymerase epitopes during and after acute viral hepatitis. *J. Exp. Med.* 181:1047–1058.
38. Vitiello, A., D. Marchesini, J. Furze, L.A. Sherman, and R.W. Chesnut. 1991. Analysis of the HLA-restricted influenza-specific cytotoxic T lymphocyte response in transgenic mice carrying a chimeric human–mouse class I major histocompatibility complex. *J. Exp. Med.* 173:1007–1015.
39. Yewdell, J.W., and J.R. Bennink. 1989. Brefeldin A specifically inhibits presentation of protein antigens to cytotoxic T lymphocytes. *Science*. 244:1072–1075.
40. van Endert, P.M., R. Tampé, T.H. Meyer, R. Tisch, J.-F. Bach, and H.O. McDevitt. 1994. A sequential model for peptide binding and transport by the transporters associated with antigen processing. *Immunity*. 1:491–500.
41. Bertoletti, A., F.V. Chisari, A. Penna, S. Guilhot, L. Galati, G. Missale, P. Fowler, H.J. Schlicht, A. Vitiello, R.C. Chesnut, et al. 1993. Definition of a minimal optimal cytotoxic T-cell epitope within the hepatitis B virus nucleocapsid protein. *J. Virol.* 67:2376–2380.
42. Hammond, S.A., R.C. Bollinger, T.W. Tobery, and R.F. Silliciano. 1993. Transporter-independent processing of HIV-1 envelope protein for recognition by $CD8^+$ T cells. *Nature*. 364:158–161.
43. Zweerink, H.J., M.C. Gammon, U. Utz, S.Y. Sauma, T. Harrer, J.C. Hawkins, R.P. Johnson, A. Sirotina, J.D. Hermes, B.D. Walker, et al. 1993. Presentation of endogenous peptides to MHC class I-restricted cytotoxic T lymphocytes in transport deletion mutant T2 cells. *J. Immunol.* 150:1763–1771.
44. Schumacher, T.N., M.L. De Bruijn, L.N. Vernie, W.M. Kast, C.J. Melief, J.J. Neefjes, and H.L. Ploegh. 1991. Peptide selection by MHC class I molecules. *Nature*. 350:703–706.
45. Anton, L.C., J.W. Yewdell, and J.R. Bennink. 1997. MHC class I-associated peptides produced from endogenous gene products with vastly different efficiencies. *J. Immunol.* 158: 2535–2542.
46. Sykulev, Y., M. Joo, I. Vturina, T.J. Tsomides, and H.N. Eisen. 1996. Evidence that a single peptide-MHC complex on a target cell can elicit a cytolytic T cell response. *Immunity*. 4:565–571.
47. Pamer, E.G., A.J. Sijts, M.S. Villanueva, D.H. Busch, and S. Vijh. 1997. MHC class I antigen processing of *Listeria monocytogenes* proteins: implications for dominant and subdominant CTL responses. *Immunol. Rev.* 158:129–136.
48. Yewdell, J.W., and J.R. Bennink. 1999. Immunodominance in major histocompatibility complex class I-restricted T lymphocyte responses. *Annu. Rev. Immunol.* 17:51–88.
49. Chen, Y., J. Sidney, S. Southwood, A.L. Cox, K. Sakaguchi, R.A. Henderson, E. Appella, D.F. Hunt, A. Sette, and V.H. Engelhard. 1994. Naturally processed peptides longer than nine amino acid residues bind to the class I MHC molecule HLA-A2.1 with high affinity and in different conformations. *J. Immunol.* 152:2874–2881.
50. Hughes, E.A., B. Ortmann, M. Surman, and P. Cresswell. 1996. The protease inhibitor, *N*-acetyl-L-leucyl-L-leucyl-leucyl-L-norleucinal, decreases the pool of major histocompatibility complex class I–binding peptides and inhibits peptide trimming in the endoplasmic reticulum. *J. Exp. Med.* 183:1569–1578.
51. Falk, K., O. Rotzschke, S. Stevanovic, G. Jung, and H.G. Rammensee. 1991. Allele-specific motifs revealed by sequencing of self-peptides eluted from MHC molecules. *Nature*. 351:290–296.
52. Lammert, E., D. Arnold, M. Nijenhuis, F. Momburg, G.J. Hammerling, J. Brunner, S. Stevanovic, H.G. Rammensee, and H. Schild. 1997. The endoplasmic reticulum-resident stress protein gp96 binds peptides translocated by TAP. *Eur. J. Immunol.* 27:923–927.