THE JOURNAL OF BIOLOGICAL CHEMISTRY
© 1995 by The American Society for Biochemistry and Molecular Biology, Inc.

Vol. 270, No. 12, Issue of March 24, pp. 6515–6522, 1995
Printed in U.S.A.

Presentation without Proteolytic Cleavage of Endogenous Precursors in the MHC Class I Antigen Processing Pathway*

(Received for publication, November 17, 1994, and in revised form, January 13, 1995)

Daniel Buchholz‡, Paul Scott§, and Nilabh Shastri¶

From the Department of Molecular and Cell Biology, Division of Immunology, University of California, Berkeley, California 94720-3200

The antigen presentation pathway yields peptide-MHC class I complexes on the antigen presenting cell (APC) surface for recognition by appropriate T-cells. Expression of the peptide-MHC complex on APC surface is preceded by several steps that include the generation of peptide fragments in the cytoplasm and their assembly with MHC molecules in the endoplasmic reticulum. It is now clear that MHC binding to optimally processed peptides in the endoplasmic reticulum is obligatory for their stable expression on the cell surface. However, whether a similar obligatory relationship exists between generation of processed peptides and their expression as peptide-MHC on APC surface is not known. Here, we addressed this question by analyzing the processing of ovalbumin (aa257–264, SL8) or influenza nucleoprotein (aa366–374, AM9) analogs. We examined the generation of naturally processed peptides using precursors that did, or did not, contain residues flanking the optimal MHC-binding peptides. By characterizing the peptides generated from these precursors by T-cell stimulation assays and by high performance liquid chromatography analysis, we established that intracellular assembly of peptide-MHC complexes and their expression on the cell surface can occur with peptides that lack flanking residues. The presentation of these endogenously synthesized perfect fit peptides demonstrates that the cleavage of precursor polypeptides is an independent step in the antigen presentation pathway.

Antigen presentation is the mechanism by which peptide-MHC[1] class I complexes are displayed on the cell surface for recognition by appropriate T-cells (1, 2). The peptides displayed by MHC on the cell surface represent precisely cleaved proteolytic fragments of intracellular proteins (3, 4). From the analysis of naturally processed peptides, a clear picture of the general structural features of these peptide products (5) and how they bind to the MHC molecules has emerged (6, 7). This taken together with discoveries of the transporter and proteasome genes within the MHC has suggested an outline of how endogenous proteins yield peptide-MHC complexes on the cell surface (8).

At least four distinct events are necessary for generating peptide-MHC complexes on the APC surface. (*a*) Intracellular proteins are fragmented, and their peptide products are (*b*) transported into the ER wherein (*c*) they are assembled into peptide-MHC complexes that (*d*) travel to their final destination on the cell surface (8–10). The constitutive nature and remarkable efficiency of this process (11–15) raises the question of whether these steps occur independently, or whether each step is dependent upon successful completion of the preceding step, *i.e.* the peptide-MHC complexes are generated by a series of concerted steps. Indeed, the last three steps (*b*-*d*) of this pathway are concerted. Loss of either one or both subunits of the TAP1/2 transporter prevents assembly and surface expression of peptide-MHC complexes (16–20). Likewise, empty MHC class I molecules are physically associated with the TAP heterodimer as well as with calnexin and are retained in the ER until they bind peptides (21–23). Thus, peptide transport, assembly of peptide-MHC complexes, and their expression on the cell surface are sequential and concerted steps. Because all known naturally processed peptides represent proteolytic products of cellular proteins, existence of a similar obligatory relationship, if any, between the requirements for proteolysis and display of their products on the cell surface is not known. For example, it is conceivable that, under physiological conditions, TAP may transport only those endogenous peptides that were produced by the proteasome.

Current evidence favors the 26S proteasome, containing the MHC-encoded (24–27), γ-interferon-inducible (28, 29) LMP-2 and LMP-7 polypeptides, as being responsible for antigen processing (8, 10). Whether this is the only mechanism for generating processed peptides has been questioned first by the intact antigen presenting ability of cell lines lacking both LMP-2 and LMP-7 (30–32), and more recently by the dramatically distinct phenotypes of peptide-MHC expression between TAP1 knock-out mice (20) and LMP-7 (33) or LMP-2 knock-out mice (34). TAP1 mutant mice are virtually devoid of MHC molecules on the cell surface, fail to present endogenous peptide-MHC class I complexes, and as a consequence are severely depleted in their CD8+ T-cell subset in the thymus and periphery (20, 35, 36). By contrast, LMP-7 or LMP-2 mutant mice express significant to normal levels of MHC and CD8+ T-cells and show only selective defects in antigen presentation to T-cells (33, 34). Whether the incomplete defects in peptide-MHC expression in LMP-2 and LMP-7 mutant mice can be attributed to a reduced or abnormal supply of naturally processed peptides and/or to inefficient peptide transport by disruption of LMP-2 and/or LMP-7-dependent link between the proteasome and the TAP complex is presently unclear. Thus, while it is established that efficient supply of cytoplasmic peptides to the MHC in the ER

* This research was supported by National Institutes of Health Grant AI-26604 and by a grant from the UC Tobacco Related Disease Research Grant (to N. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Recipient of a pre-doctoral fellowship from the Howard Hughes Medical Institute.

§ Recipient of an University of California System-wide Biotechnology Research and Education Program Training Grant.

¶ To whom correspondence should be addressed: Dept. of Molecular Cell Biology LSA 421, University of California, Berkeley, CA 94720-3200. Tel.: 510-643-9197; Fax: 510-643-9230.

[1] The abbreviations used are: MHC, major histocompatibility complex; APC, antigen presenting cell; ER, endoplasmic reticulum; PBS, phosphate-buffered saline; HPLC, high performance liquid chromatography.

depends upon proteolysis (37), as well as TAP-mediated transport (20), the mechanism(s) that generate naturally processed peptides and deliver them to TAP remain poorly understood.

A different approach to addressing the role of proteolysis in antigen presentation is to focus on the antigen rather than the protease(s). By examining the processed products of antigen precursors that do or do not contain residues flanking the optimal MHC-binding ("perfect-fit") peptides, we can determine whether peptide cleavage had occurred and whether it was an obligatory step in the expression of peptide-MHC complexes on the cell surface. Although conceptually straightforward, this approach has been difficult to implement. First, only vanishingly small amounts of processed peptides are present in the APC, and individual peptides are detectable in complex cell extracts only because of highly sensitive T-cell activation assays (3, 4, 38). Second, a more profound problem is the fact that efficient synthesis of precursor polypeptide requires presence of the translational initiation ("ATG," methionine) codon (15, 39). Thus, endogenously synthesized perfect-fit precursors must contain methionine as the first residue of the processed peptide. Recently, we showed that cells expressing minimal analogs of the ovalbumin octapeptide, aa257–264 (SIINFEKL, SL8), serve as efficient APCs for SL8/K^b-specific T-cells as well as allow analysis of the processed peptides in HPLC-fractionated cell extracts (14, 40). By comparing extracts of cells expressing different MHC molecules, we showed that appropriate MHC molecules are essential for stabilizing otherwise rapidly degraded processed peptides. This extreme instability of processed peptides ruled out their direct analysis in cells lacking MHC. However, we noticed one instance where the Met-SL8 (MSL8) peptide was actually present in D^b cells expressing the minigene encoding the MSL8 precursor. The fortuitous discovery of the perfect-fit MSL8 peptide that could be endogenously translated as well as be detected as such in cell extracts prompted us to use this model system to address the question of whether cleavage of flanking residues from endogenously synthesized polypeptides is an obligatory step in the antigen presentation pathway.

We compared the naturally processed peptides that were generated from N terminally extended precursors or from perfect fit peptides. DNA constructs encoding ovalbumin (OVA257–264, SL8) or influenza nucleoprotein (NP366–374, AM9) analogs were used as model antigens for generating peptide/K^b or D^b MHC complexes. MHC-bound peptides were extracted from transfected cells and were characterized by their HPLC elution profiles. Here we show that sequences flanking the optimal MHC-binding peptides were unfailingly cleaved from endogenously synthesized precursors indicating that they were processed. However, perfect fit precursors that exactly matched the optimal MHC-binding peptides were presented on the cell surface as such with comparable efficiency. These results show that cleavage of flanking residues is not obligatory for the intracellular assembly or for cell surface expression of peptide-MHC complexes and establish that proteolytic cleavage of antigenic precursors can be segregated from other steps in the antigen presentation pathway.

MATERIALS AND METHODS

Antibodies, Antigens, and Plasmids—Y3 (anti-K^b) and B22.249 (anti-D^b) were from the American Type Culture Collection and kindly provided by Dr. David Raulet (University of California, Berkeley), respectively. Synthetic peptides were prepared by automated solid-phase synthesis and provided by Dr. David King (UC, Berkeley) and Dr. J. Kobori (California Institute of Technology) (14). The sequences in the single-letter code were SIINFEKL (SL8), MIINFEKL (ML8), IILFEKL (IL7), ILFEKL (IL6), MSIINFEKL (MSL8), MKSIINFEKL (MKSL8), MMSIINFEKL (MMSL8), ASNENMETM (AM9), and MSNENMETM (MM9). Plasmid constructs encoding peptides of indicated sequence were made by cloning synthetic oligonucleotides into the *BstXI* and *XbaI* sites of the pcDNA1 vector (Invitrogen) as described earlier (14). All constructs were confirmed by nucleotide sequencing.

Cell Lines—*LacZ*-inducible, SL8/K^b-specific B3Z and AM9/D^b-specific DBFZ.25 T cell hybrids have been described (14, 41, 42). The ML8/K^b-specific BKMZ T cells were obtained by *in vitro* immunization with ML8 peptide.[2] K^b-COS, D^b-COS, and K^b-L cells have been described (14, 41, 42), and D^b-L cells were obtained similarly. Stable K^b-L cell transfectants were obtained by co-transfecting the MSL8 or ML8 encoding DNAs with pMC1-neo^r plasmid (Stratagene) as a selection marker by the calcium phosphate method, and stable MAM9- or MM9-expressing D^b-L cells were obtained similarly (14). G418^r transfectants were cloned by limiting dilution and screened for expression of peptide-MHC complexes by T-cell stimulation assay. Cells were maintained in RPMI 1640 with 10% fetal bovine serum, 2 mM glutamine, 1 mM pyruvate, 50 μM β-mercaptoethanol, 200 units/ml penicillin, 200 μg/ml streptomycin at 37 °C in 5% CO_2/humidified air.

DNA Transfections—K^b- or D^b-COS cells were transiently transfected with plasmids using the DEAE-dextran method. DNA titrations in 96-well plates for assaying APC function have been described (14). For large scale transfections, 1 μg/ml plasmid DNA in 100 μg/ml DEAE-dextran and 100 μM chloroquin phosphate in RPMI 1640 with 10% NU-serum (Collaborative Research) was added to 1–3 × 10^6 adherent cells in a T25 or T75 flask. After 2 h, the cells were incubated with 10% Me_2SO in PBS for 2 min and returned to complete medium. After 2–3 days, cells were either assayed directly for expression of peptide-MHC on the surface (endogenous peptide assay) or used for extracting the naturally processed peptides.

Peptide Assays—The antigen/MHC-specific T cell response was measured as the β-galactosidase activity induced in the T cells following T-cell receptor occupancy (14, 42, 43). 3–10 × 10^4 T-cell hybrids were co-cultured with appropriate APC overnight. The β-galactosidase activity induced in the T cells was then measured by addition of the substrate chlorophenol red β-galactoside. After 4 h, the absorbance of the cleaved chlorophenol red in the wells was measured at 595 nm with 635 nm as the reference wavelength. To detect endogenously synthesized peptide-MHC complexes (endogenous peptide assay), cells transfected with DNAs encoding the antigen were used as APCs. To quantitate synthetic peptides or antigenic peptides present in peptide extracts (exogenous peptide assay), T-cells were co-cultured with either K^b- or D^b-expressing L-cells in the presence of synthetic or extracted peptides. Endogenous and exogenous peptide assay experiments were repeated at least three times, and typically the sensitivity of the assays was about 1 pM with synthetic standards.

Peptide Extraction—Total cellular peptide extractions were achieved by lysing cells in 0.1% trifluoroacetic acid in water as described (40, 44). For extraction of total MHC-bound peptides, 1.2 ml of ice-cold lysis buffer (20 mM Tris-HCl, pH 8, 150 mM NaCl, 1% Nonidet-40, 10 mM EDTA, 0.01% NaN_3, 5 mg/ml bestatin, 200 mM puromycin, 1 mg/ml pepstatin A, 10 mM 1-chloro-3-tosylamido-7-amino-2-heptanone, 1 mg/ml aprotinin, 500 mM iodoacetamide, 0.1 M phenylmethylsulfonyl fluoride) was added to 1–2 × 10^6 washed cells. The lysate was incubated on ice for 5 min and centrifuged to pellet cell debris. 10–20 μl of anti-MHC antibody ascites and protein A-Sepharose beads (Sigma) were added to supernatant fluid and rocked for 1.5 h at 4 °C. Peptides were eluted from washed beads with 0.5 ml 0.1% trifluoroacetic acid and filtered through a 10-kDa cutoff filter (Millipore, Bedford, MA). The filtrate was dried in a vacuum centrifuge (Savant Instruments, Inc., Farmingdale, NY). Samples were resuspended in PBS or medium and used for HPLC analysis.

For separation of peptides associated with intracellular and cell surface MHC molecules, cells were first incubated with B22.249 (anti-D^b) ascites and thoroughly washed before lysis. The supernatant fluid of the centrifuged lysate was added to protein A-Sepharose beads to isolate cell surface MHC molecules. The supernatant fluid of the lysate containing intracellular MHC molecules was then subject to another round of anti-D^b immunoprecipitation. Peptides from these MHC immunoprecipitates were eluted and assayed as described above. Data shown are representative of three independent experiments.

HPLC Analysis—Samples of peptide extracts were redissolved in PBS and fractionated on an Hewlett Packard 1050 quaternary pump HPLC using a 4.6 × 250-mm Vydac C18 column with 5-μm particle size and 300 Å pores. For the SL8 family of peptides, the gradient began at 77% of 0.1% trifluoroacetic acid in water and 23% of 0.1% trifluoroacetic acid in acetonitrile and increased to 38% of 0.1% trifluoroacetic acid in

[2] P. Scott, unpublished results.

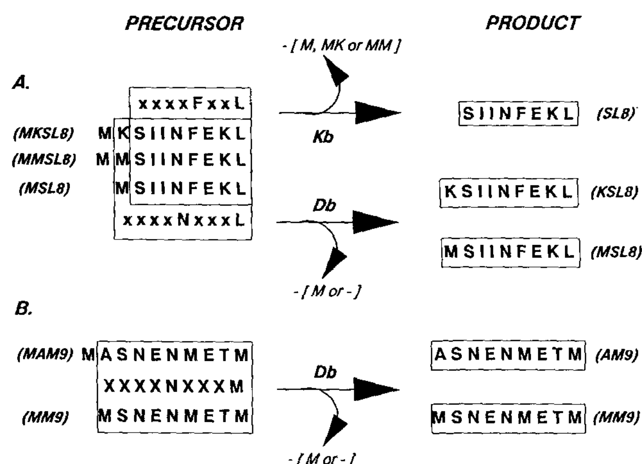

FIG. 1. **Schematic diagram showing the precursor-product relationships for the antigens and MHC molecules used.** Precursor peptides and their abbreviations are shown on the left with *boxed* peptides representing the products associated with the K^b or D^b MHC class I molecules. One, two, or no N-terminal amino acids are cleaved to result in the peptide products shown with their abbreviations on the right. Peptide sequences with xs represent the MHC allele-specific consensus motifs for bound peptides. *A*, SL8/K^b-specific B3Z T cell hybrids were used to analyze the depicted precursor-product model systems. *B*, AM9 or MM9/D^b-specific DBFZ.25 T cell hybrids were used to analyze this depicted precursor/product model system.

acetonitrile at the rate of 0.5%/min. The same gradient was used to separate NP peptide analogs but began at 95% of 0.1% trifluoroacetic acid in water and 5% of 0.1% trifluoroacetic acid in acetonitrile. One-ml fractions were collected, dried in a vacuum centrifuge, resuspended in PBS, and used in the exogenous peptide assay. Mock runs of 0.1% trifluoroacetic acid in water were performed and assayed between each sample run to demonstrate absence of peptide carry over from previous runs. These experiments were repeated 2–10 times, and the HPLC profiles were extremely reproducible with less than 0.5 min variation between runs.

RESULTS

Generation of Cleaved SL8/K^b Complex from Precursor Polypeptides—In K^b APC, processing of the 386 aa ovalbumin protein yields the processed octapeptide OVA257–264 (SL8) (14, 45, 46). We have recently established that naturally processed SL8 can be detected in HPLC-fractionated trifluoroacetic acid extracts of cells transfected with minimal SL8 precursors such as Met-SL8 (MSL8) or Met-Lys-SL8 (MKSL8) (Fig. 1*A*; Ref. 40). This study showed that sequences (Met or Met-Lys) flanking the K^b motif-bearing octapeptide SL8 were removed by a proteolytic step of the antigen processing mechanism. Interestingly, we also found that in cells expressing the D^b MHC molecules, the processing of the same precursors yielded nonapeptides (KSL8 or MSL8) that contained an additional residue flanking the SL8 peptide in the trifluoroacetic acid extracts (Fig. 1*A* in Ref. 40). Thus, as judged by analysis of trifluoroacetic acid extracts, residues flanking the optimal peptides in each of the precursors tested were consistently and reproducibly removed.

Peptide-MHC complexes expressed on the cell surface are first assembled in the ER (47), and the possibility remained that processed peptides in the trifluoroacetic acid extracts represented only a subset of processed peptides (*e.g.* only those peptides present on the cell surface). To establish that all extra- and intracellular MHC-bound peptides had been cleaved to the optimal SL8 peptide, we looked for possible SL8 analogs that could be bound to K^b MHC in cells lysed with detergents. First, synthetic SL8, KSL8, or MSL8 peptides were all capable of stimulating SL8/K^b-specific B3Z T-cells (Fig. 2*A*). The dose-response curves, consistently within 2-fold of each other, show that picomolar concentrations of these peptides could be readily detected, and in addition, each of these peptides could also be readily distinguished by their characteristic HPLC elution profiles (Fig. 2*B*). Second, both MSL8 and MKSL8 DNA constructs allowed generation of T-cell-stimulating peptide/K^b complexes on the cell surface (Fig. 2*C*). Nevertheless, with both precursors only the cleaved SL8 octapeptide was bound by K^b MHC (Fig. 2*D*). The HPLC elution profiles of T-cell stimulating activity of naturally processed peptides in anti-K^b (Y3 monoclonal) immunoprecipitates from either MSL8- or MKSL8-transfected cell extracts corresponded exactly to that of synthetic SL8. Other possible candidates such as MSL8 or KSL8 peptides were clearly undetectable and were estimated to represent less than 0.1% of the amount of SL8 recovered. In other experiments (data not shown), the same profiles were obtained when K^b MHC were immunoprecipitated with polyclonal antiserum specific for the cytoplasmic tail of K^b (48), ruling out a possible peptide-specific bias in the subset of K^b MHC immunoprecipitated by the Y3 mAb (49, 50). We conclude that in living cells, similar to the native ovalbumin, the same SL8 octapeptide was processed from these 9–10 aa precursors for presentation by K^b MHC. However, SL8 itself cannot be synthesized within cells without the addition of the methionine residue for initiating translation (15). Therefore, to test whether this proteolytic removal of N-terminal-flanking residues, regardless of the specific mechanism involved, was obligatory for generation of peptide-MHC complexes, we analyzed model precursors that exactly matched the MHC-bound, naturally processed peptides and could be translated within cells.

MSL8 Peptide Achieves Cell Surface Expression as a MSL8-D^b Complex without Processing—We had observed earlier that the MSL8 nonapeptide was present in total trifluoroacetic acid extracts of D^b cells transfected with the MSL8 construct (40). However, because T-cells specific for MSL8/D^b complexes are not available, it remained unclear whether the MSL8 peptide was physically bound to D^b and whether it was present on the cell surface as a MSL8-D^b complex. To resolve these possibilities, D^b-COS cells were transfected with MSL8 or MMSL8 constructs to provide precursors that were either a perfect-fit (MSL8) or contained an additional Met residue (MMSL8) flanking the D^b consensus motif (see Fig. 1*A*). By sequential immunoprecipitations, the D^b MHC molecules were separated into those present on the cell surface *versus* those present intracellularly (see "Materials and Methods"). The co-immunoprecipitated peptides were then eluted with trifluoroacetic acid and analyzed with SL8/K^b-specific B3Z T-cells. MSL8 cells produced amounts of T-cell stimulating peptides from intracellular or cell surface D^b molecules comparable to amounts recovered from cells expressing MMSL8 precursors (Fig. 3*A*). Interestingly, the amount of intracellular peptide was about 3-fold higher than that recovered from the cell surface perhaps reflecting the known slow rate of peptide/D^b assembly and/or transport (23). Most importantly, the activity in the trifluoroacetic acid extracts represented predominantly the MSL8 peptide as shown by its identical HPLC elution profile with synthetic MSL8 peptide (Fig. 3*B*). No MMSL8 was detected in the extracts. The small amount of activity (<2%) detected as SL8 is most likely due to post lysis degradation because this activity was only observed in whole cell trifluoroacetic acid extracts, but not in extracts of immunoprecipitated molecules (data not shown). The generation of MSL8/D^b complexes on the cell surface from MSL8 precursor strongly suggests that cleavage of flanking residues is not obligatory for entry into the antigen processing pathway. Moreover, because comparable amounts of cleaved (from MMSL8 transfectants) or uncleaved (from MSL8 transfectants) MSL8 peptide were re-

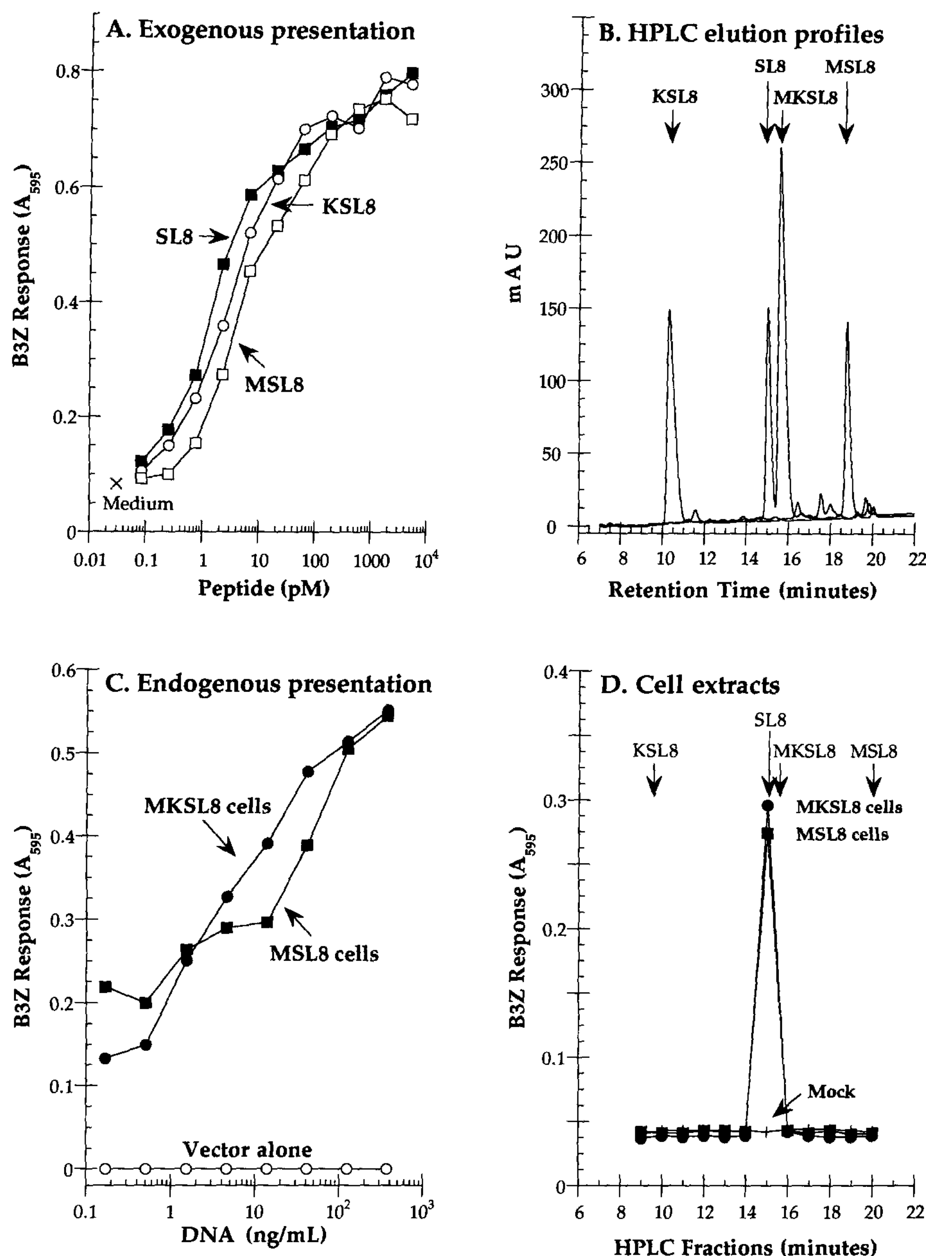

FIG. 2. **Only cleaved products are present in K^b-COS cells transfected with precursor constructs encoding N terminally extended antigenic peptides.** *A*, K^b-L cells with synthetic peptides, SL8 (■), KSL8 (○), and MSL8 (□), at the indicated concentrations were assayed in the exogenous peptide assay to show that these peptides are efficiently recognized. Medium control (×). *B*, HPLC chromatograms of the synthetic peptides, KSL8, SL8, MKSL8, and MSL8, show excellent resolution of KSL8, SL8, and MSL8. *C*, K^b-COS cells were transiently transfected with vector DNA (○) or DNA constructs encoding MSL8 (■) or MKSL8 (●) at the indicated DNA concentrations. Stimulatory peptide-MHC complex expression in transfected K^b-COS cells was shown by the endogenous peptide assay. *D*, precursor peptides are cleaved to SL8 in K^b-COS cells as shown by recovery of only SL8 in HPLC fractions of total K^b-bound peptide extracts from MSL8 (■) and MKSL8 (●) transfected K^b-COS cells and assay of the fractions in the exogenous peptide assay. Mock run (+) shows absence of any peptide carryover from previous runs. One of five similar experiments is shown for each panel.

covered in the extracts also suggests that the cleavage of a single flanking residue is not a rate-limiting step in the antigen presentation pathway.

ML8 and MM9 Peptides Yield Cell Surface Peptide-MHC Complexes without Processing—To establish the generality of the above biochemical analysis, we tested two additional model systems where the presence of endogenously synthesized perfect fit peptide-MHC complexes on APC surface could be directly assayed with T-cells. This was particularly important because T-cell activation is by far the most sensitive indicator for the existence of peptide-MHC complexes on the cell surface (14, 15). As the second model, we used the optimal octamer, ML8 (MIINFEKL) peptide as a perfect-fit, endogenously synthesized peptide for generating the ML8-K^b complex. BKMZ T-cells recognize both ML8-K^b and SL8-K^b complexes (Fig. 4*A*), yet B3Z T cells recognize only the SL8-K^b complex (Fig. 4*B*). The hepta- or hexapeptides, IL7 (IINFEKL) or IL6 (INFEKL), lacking one or two N-terminal residues were 100-1000-fold less active in stimulating both T-cells. To obtain endogenous presentation, K^b-L cells were stably transfected with either ML8 (ML8/K^b-L) or MSL8 (MSL8/K^b-L) DNA constructs. Significantly, both MSL8- and ML8-expressing K^b transfectants were comparable in their ability to present peptide-MHC complexes to BKMZ T cells (Fig. 4*C*). This shows that K^b complexes were generated with both the processed SL8 product from the MSL8 precursor (see Fig. 2) and with the unprocessed ML8 itself. Given that shorter seven- or six-mer peptides are poorly transported by the TAP complex (51–54) and are also poorly bound by K^b MHC (55), it is unlikely that a cleaved, shorter peptide such as IL7 or IL6, was actually presented on the APC surface. Moreover, that B3Z T cells do recognize the shorter IL7 and IL6 peptides as well as BKMZ cells but fail to recognize ML8/K^b-L cells (Fig. 4*D*) strongly suggests that ML8 itself was presented on the cell surface rather than a cleaved product such as IL7.

As the third and completely independent model, we used T-cells specific for the influenza nucleoprotein peptide AM9 (ASNENMETM)-D^b complex (Fig. 1*B*). The DBFZ.25 T-cells recognize both AM9 and MM9 (MSNENMETM) synthetic peptides with superimposable dose-response curves indicating that the Ala to Met substitution does not affect D^b binding or T-cell

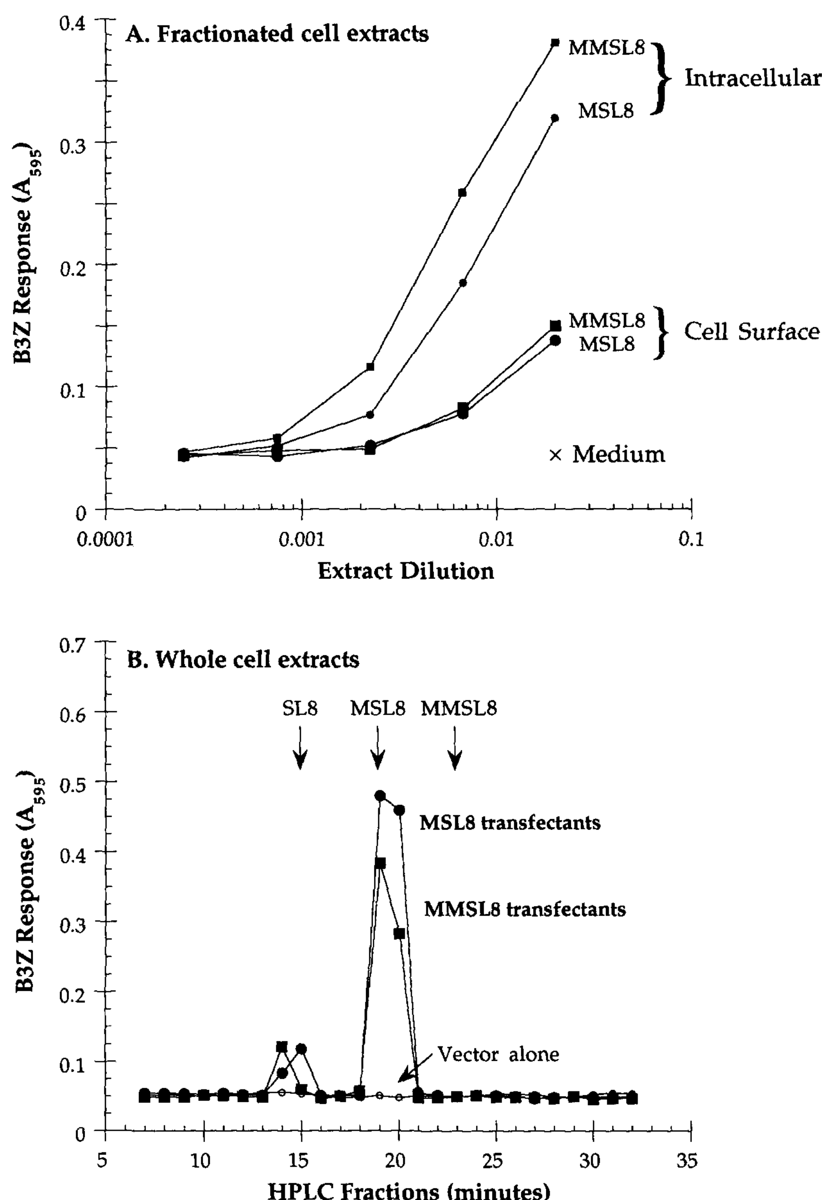

FIG. 3. **Uncleaved MSL8 is expressed on the cell surface of D^b-COS cells.** D^b-COS cells were transfected with vector DNA (○) or DNA constructs encoding MSL8 (●) or MMSL8 (■). *A*, stimulatory peptides present on cell surface (*large symbols*) or intracellular (*small symbols*) MHC molecules were acid eluted and detected in the exogenous peptide assay. Medium control (×). *B*, identification of stimulatory peptides as cleaved MMSL8 or uncleaved MSL8 was achieved by HPLC fractionation of total MHC-bound peptide extracts from MMSL8 (■) and MSL8 (●) transfected D^b-COS cells and assay of the fractions in the exogenous peptide assay. One of two to three similar experiments is shown for each panel.

receptor specificity for the peptide-D^b complexes (Fig. 5*A*). Because the presence of methionine as the first residue allows translation, D^b-COS cells were transfected with DNA constructs encoding MM9 or MAM9 and were tested with DBFZ.25 T-cells. Significantly, the T-cell response to cells transfected with either DNA construct was superimposable suggesting that comparable amounts of peptide-D^b complexes were expressed on the cell surface (Fig. 5*C*). The naturally processed peptides from D^b-COS cells expressing either MAM9 or MM9 peptides were extracted and analyzed by HPLC. This was important to establish their identity as well as to test the formal possibility that both MAM9 and MM9 were giving rise to a common cleaved peptide fragment (*e.g.* the octamer SNENMETM) that was responsible for stimulating the DBFZ.25 T-cells. The HPLC elution profiles of extracted peptides produced in either MAM9- or MM9-transfected cells yielded single activity peaks with distinct retention times (Fig. 5*D*). Furthermore, the elution profiles of these peaks were identical to those obtained with synthetic AM9 and MM9 peptides (compare Figs. 5, *B* and *D*). Similar results were also obtained with D^b-L cells stably transfected with MAM9- or MM9-encoding DNA constructs ruling out any potential artifacts in the transient COS cell expression system (data not shown). Thus, as observed above for the MSL8-D^b and the ML8-K^b complexes, the MM9 peptide also entered the antigen processing pathway to yield the MM9-D^b complex on the cell surface. In summary, our data unequivocally show that cleavage of flanking residues that necessarily occurs for all known cellular proteins is not obligatory for peptide-MHC expression on the cell surface.

DISCUSSION

The display of precisely cleaved peptides from cellular proteins has emerged as a defining feature of the peptide-MHC class I presentation pathway. Here, we establish for the first time that the precise cleavage of flanking residues that invariably occurs during the generation of optimal MHC-binding peptides is not obligatory for presentation of endogenously translated precursors.

Several key steps regulate the MHC class I antigen presentation pathway. The best characterized of these is the translocation of antigenic peptides from the cytoplasm to the ER by the TAP1/TAP2 (TAP) transporter. Compelling evidence is now available that TAP-mediated translocation of synthetic peptides into the ER is ATP-dependent and is selective for peptide

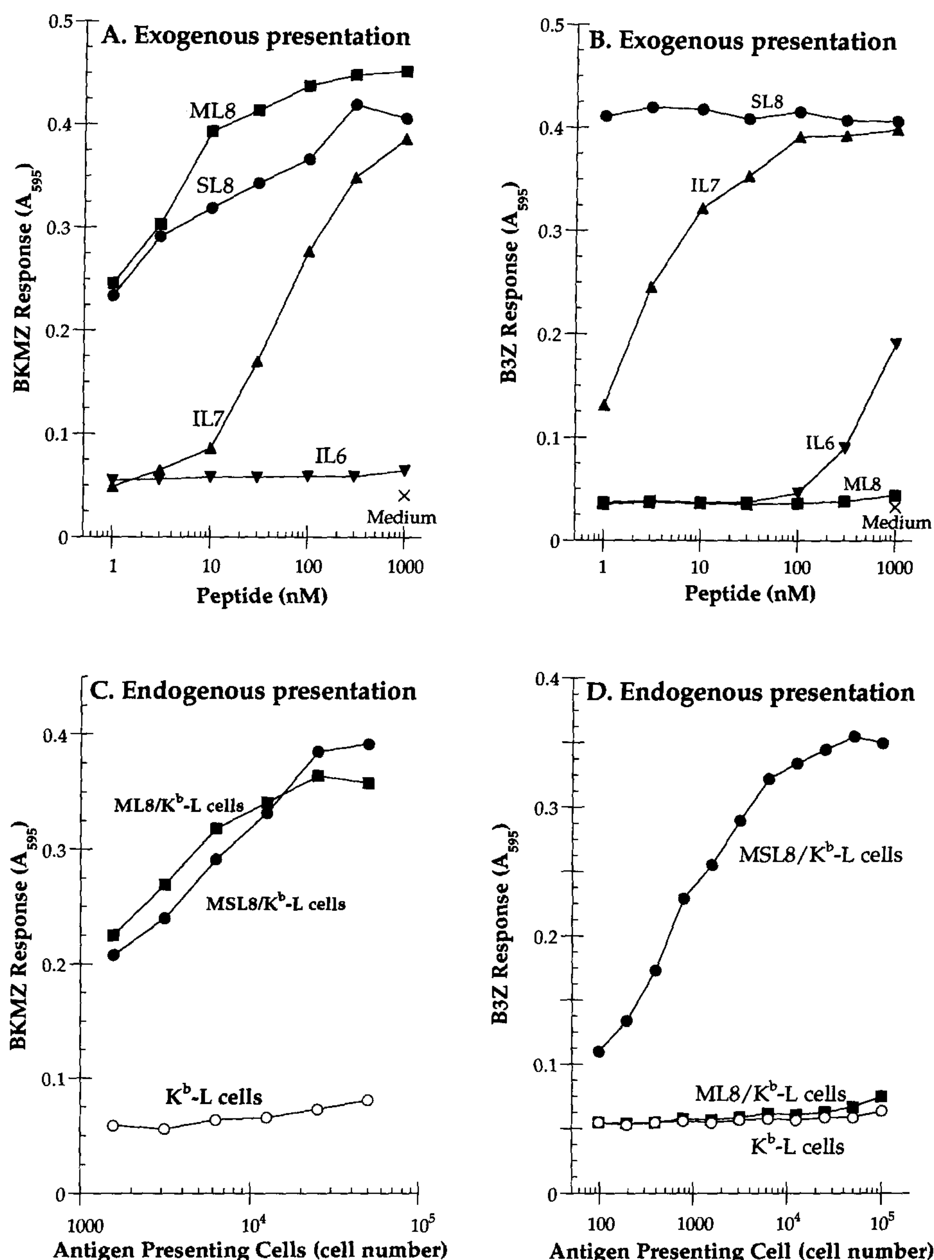

FIG. 4. **Uncleaved ML8 octapeptide is expressed on the cell surface of K^b-expressing L cells.** *A* and *B*, K^b-L cells with synthetic peptides ML8 (■), SL8 (●), IL7 (▲), and IL6 (▼) at the indicated concentrations were assayed in the exogenous peptide assay. Medium control (×). BKMZ T cells (*A*) recognize ML8 and SL8, whereas B3Z T cells (*B*) do not recognize ML8. The truncated peptides are recognized less efficiently by both T cells. *C* and *D*, K^b-L cells (○) or K^b-L cells stably transfected with ML8 (■) or MSL8 (●) constructs were used in the endogenous peptide assay. BKMZ T cells (*C*) recognize both ML8- and MSL8-expressing K^b-L cells, but B3Z T cells (*D*) recognize only MSL8-expressing K^b-L cells. One of three similar experiments is shown for each panel.

length (between 8–25 residues) and upon the nature of the C-terminal residue (20, 51–54). Loss of TAP function, with a few exceptions (56–58), effectively disrupts assembly of peptide/MHC complexes in the ER and as a consequence causes dramatic loss of the ability to present endogenous peptide-MHC complexes on the cell surface (19, 20, 57, 59). Because endogenous peptide-MHC class I expression in the thymus is essential for positive selection, TAP knock-out mice also lack CD8+ T-cells (20). *In vitro* assays have shown that TAP binds synthetic peptides (54) and is associated with empty MHC class I molecules (21, 22), thus providing the structural basis for peptide transport and for efficient loading of these peptides onto MHC molecules in the lumen of the ER. In addition to physical association with TAP, empty MHC molecules interact with the chaperone calnexin in the ER that regulates assembly of MHC heavy chains with β2-microglobulin and their exit from the ER (23, 60). Therefore, the availability of peptides in the ER and their loading onto the MHC molecules are key steps that, if disrupted, can severely compromise the exit of peptide-MHC complexes from the ER en route to the cell surface.

By contrast to the overwhelming evidence for the key role of TAP in the antigen presentation pathway, the mechanism by which peptides are generated and supplied to TAP remain poorly defined. The hypothesis that precisely cleaved peptides are generated by the proteasome in the cytoplasm and are the natural substrates for TAP transport remains to be proven. Clearly the 26S cytoplasmic proteasome is involved in the antigen presentation pathway as elegantly demonstrated with selective inhibitors by the Rock laboratory (37). However, whether the physiological products of this proteasome are optimal peptides as suggested by its *in vitro* activity is not yet clear (61). Indeed, MHC-associated peptides considerably longer (up to 33 aa) than the final 8–10 residue peptides have been found in cells (62–64). Although it is not known whether these longer peptides are intermediates in the antigen presentation pathway, the expression of the HLA-B27-associated peptides was shown to be TAP-dependent (63). In addition, two examples have provided evidence consistent with the notion that cleavage of longer precursors to the final products can occur in the ER (40, 65). These observations have raised questions

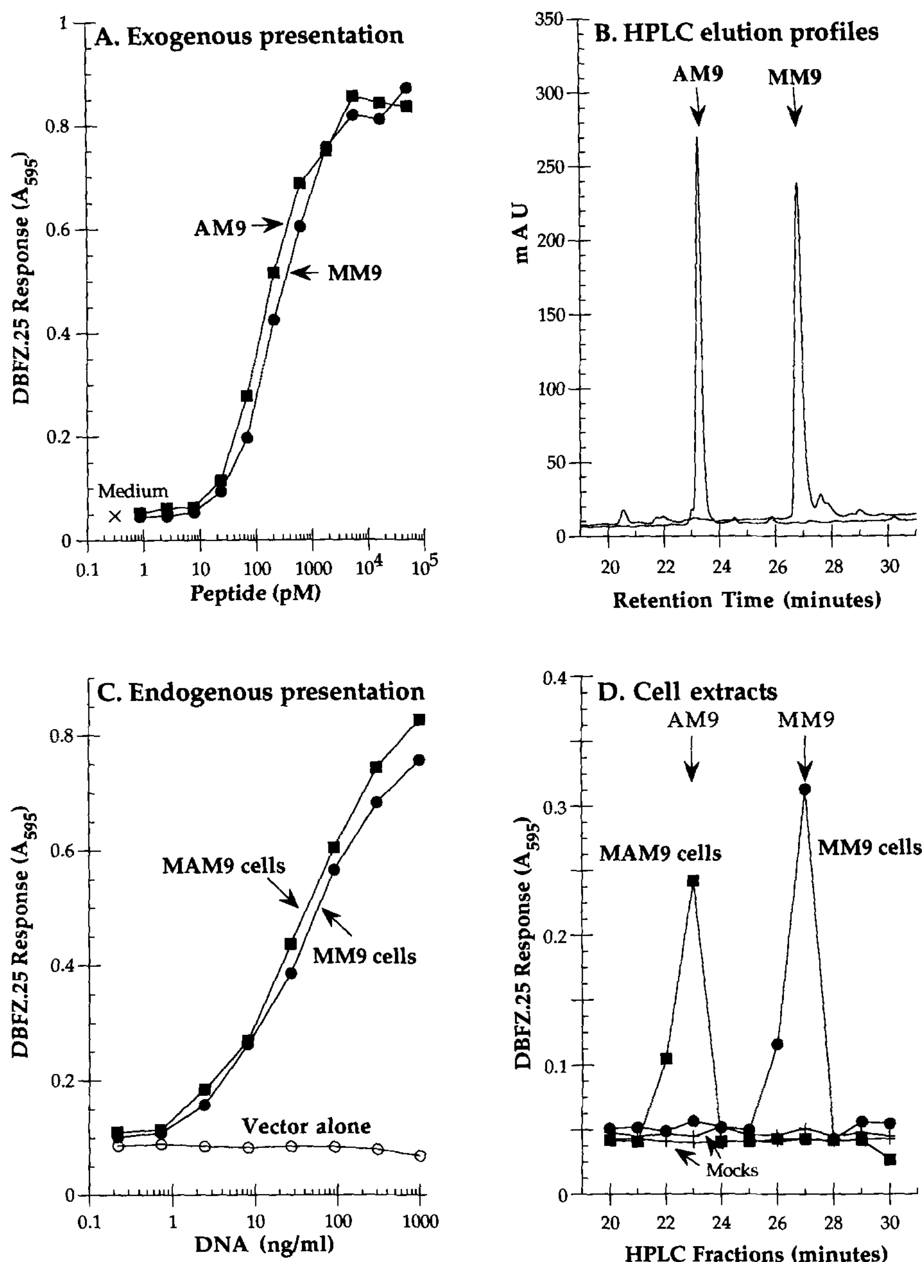

FIG. 5. **Uncleaved MM9 nonapeptide is expressed on the cell surface of D^b-COS cells.** *A*, D^b-L cells with synthetic peptides, AM9 (■) and MM9 (●), at the indicated concentrations were assayed in the exogenous peptide assay to show that both peptides were recognized by DBFZ.25 T cells. Medium control (×). *B*, HPLC chromatograms of the synthetic peptides, AM9 and MM9, show excellent resolution. *C*, D^b-COS cells were transiently transfected with vector DNA (○) or DNA constructs encoding MM9 (●) or MAM9 (■) at the indicated DNA concentrations. Stimulatory peptide/D^b expression in the transfected D^b-COS cells was indicated by comparable T cell response. *D*, identification of stimulatory peptide as cleaved MAM9 or uncleaved MM9 was achieved by HPLC fractionation of trifluoroacetic acid peptide extracts from MAM9 (■) and MM9 (●) transfected D^b-COS cells and assay of the fractions in the exogenous peptide assay. Mock runs (+) show absence of peptide carry over between samples. One of four or five similar experiments is shown for each panel.

concerning the identity of the natural TAP substrates and the temporal order of peptide cleavage steps, *i.e.* before and/or after transport. In this context, presentation of the perfect-fit peptides used here can serve as the first definitive examples of an endogenous antigen (MSL8, ML8, or MM9) that remain unmodified from synthesis to cell surface expression as peptide-MHC complexes. Furthermore, because efficient presentation of cytoplasmic peptides depends upon TAP, as shown by Bacik *et al.* (66) and in our own unpublished studies,[3] strongly suggests that TAP can transport perfect-fit peptides in living cells. It is important to emphasize, however, that while our results suggest that perfect-fit peptides are transported by TAP, the identity of processed peptides of normal cellular proteins and the cleavage events that occur before or after transport remain to be established. We emphasize that our results *do* establish that none of these cytoplasmic or ER cleavage events are obligatory for cell surface expression of the peptide-MHC complex.

The presentation of perfect-fit peptides shows that proteolytic cleavage steps can be dissociated from other concerted steps in the antigen presentation pathway. Both endogenously synthesized and cytoplasmically loaded native proteins yield peptide-MHC complexes on the cell surface. Except for the signal sequence-associated peptides (56, 57, 66) and rare exceptions (58), presentation of endogenous peptides occurs in a TAP-dependent manner. Whether a single obligatory cleavage mechanism (such as the LMP2/LMP7 containing proteasome) is the sole source of processed peptides or whether multiple mechanisms converge to feed into the subsequent transport step is an important unresolved question. That perfect-fit peptides bypass the cleavage steps for presentation on the cell surface allows the possibility that there could be more than one mechanism supplying processed peptides. It is interesting that redundancy in the proteolytic mechanisms was also indicated by the antigen presentation function of cells lacking LMP2 and/or LMP7 (30–34). On the other hand, it is formally possible that perfect-fit peptides are presented because they pass through the proteolytic mechanism but without removal of any residues. Further biochemical analysis of the fate of precursor

[3] S. Goth and N. Shastri, unpublished data.

proteins and their products is required to elucidate the nature of these peptide cleavage and delivery mechanism. The model systems described here provide the tools for such analysis.

Acknowledgments—We thank David King for peptide synthesis and Ginny Curtis (Hewlett Packard Instruments) for help with the HPLC.

REFERENCES

1. Yewdell, J. W., and Bennink, J. R. (1992) *Adv. Immunol.* **52,** 1–123
2. Germain, R. N. (1994) *Cell* **76,** 287–299
3. Van Bleek, G. M., and Nathenson, S. G. (1990) *Nature* **348,** 213–216
4. Rotzschke, O., Falk, K., Deres, K., Schild, H., Norda, M., Metzger, J., Jung, G., and Rammensee, H.-G. (1990) *Nature* **348,** 252–254
5. Rammensee, H.-G., Falk, K., and Rotzschke, O. (1993) *Annu. Rev. Immunol.* **11,** 213–244
6. Jardetzky, T. S., Lane, W. S., Robinson, R. A., Madden, D. R., and Wiley, D. C. (1991) *Nature* **353,** 326–329
7. Matsumura, M., Fremont, D. H., Peterson, P. A., and Wilson, I. A. (1992) *Science* **257,** 927–934
8. Monaco, J. J. (1992) *Immunol. Today* **13,** 173–179
9. Townsend, A., and Bodmer, H. (1989) *Annu. Rev. Immunol.* **7,** 601–624
10. Goldberg, A. L., and Rock, K. L. (1992) *Nature* **357,** 375–379
11. Townsend, A. R. M., McMichael, A. J., Carter, N. P., Huddleston, J. A., and Brownlee, G. G. (1984) *Cell* **39,** 13–25
12. Heath, W. R., Hurd, M. E., Carbone, F. R., and Sherman, L. A. (1989) *Nature* **341,** 749–752
13. Falk, K., Rotzschke, O., Stevanovic, S., Jung, G., and Rammensee, H.-G. (1991) *Nature* **351,** 290–296
14. Shastri, N., and Gonzalez, F. (1993) *J. Immunol.* **150,** 2724–2736
15. Shastri, N., Nguyen, V., and Gonzalez, F. (1995) *J. Biol. Chem.* **270,** 1088–1091
16. Townsend, A., Ohlen, C., Bastin, J., Ljunggren, H.-G., Foster, L., and Karre, K. (1989) *Nature* **340,** 443–448
17. Ljunggren, H.-G., Stam, N. J., Öhlén, C., Neefjes, J. J., Höglund, P., Heemels, M.-T., Bastin, J., Schumacher, T. N. M., Townsend, A., Kärre, K., and Ploegh, H. L. (1990) *Nature* **346,** 476–480
18. Spies, T., Bresnahan, M., Bahram, S., Arnold, D., Blanck, G., Mellins, E., Pious, D., and DeMars, R. (1990) *Nature* **348,** 744–747
19. Townsend, A., Elliott, T., Cerundolo, V., Foster, L., Barber, B., and Tse, A. (1990) *Cell* **62,** 285–295
20. van Kaer, L., Ashton-Rickardt, P. G., Ploegh, H. L., and Tonegawa, S. (1992) *Cell* **71,** 1205–1214
21. Ortmann, B., Androlewicz, M. J., and Cresswell, P. (1994) *Nature* **368,** 864–867
22. Suh, W.-K., Cohen-Doyle, M., Fruh, K., Wang, K., Peterson, P., and Williams, D. B. (1994) *Science* **264,** 1322–1325
23. Jackson, M. R., Cohen-Doyle, M. F., Peterson, P. A., and Williams, D. B. (1994) *Science* **263,** 384–387
24. Brown, G. M., Driscoll, J., and Monaco, J. J. (1991) *Nature* **353,** 355–357
25. Martinez, C. K., and Monaco, J. J. (1991) *Nature* **353,** 664–667
26. Glynne, R., Powis, S. H., Beck, S., Kelley, A., Kerr, L.-A., and Trowsdale, J. (1991) *Nature* **353,** 357–360
27. Kelly, A., Powis, S. H., Glynne, R., Radley, E., Beck, S., and Trowsdale, J. (1991) *Nature* **353,** 667–668
28. Yang, Y., Waters, J. B., Fruh, K., and Peterson, P. A. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 4928–4932
29. Akiyama, K., Yokota, K., Kagawa, S., Shimbara, N., Tamura, T., Akioka, H., Nothwang, H. G., Noda, C., Tanaka, K., and Ichihara, A. (1994) *Science* **265,** 1231–1234
30. Arnold, D., Driscoll, J., Androlewicz, M., Hughes, E., Cresswell, P., and Spies, T. (1992) *Nature* **360,** 171–174
31. Momburg, F., Ortiz-Navarrete, V., Neefjes, J., Goulmy, E., van de Wal, Y., Spits, H., Powis, S. J., Butcher, G. W., Howard, J. C., Walden, P., and Hammerling, G. J. (1992) *Nature* **360,** 174–177
32. Yewdell, J., Lapham, C., Bacik, I., Spies, T., and Bennink, J. (1994) *J. Immunol.* **152,** 1163–1170
33. Fehling, H. J., Swat, W., Laplace, C., Kuhn, R., Rajewsky, K., Muller, U., and von Boehmer, H. (1994) *Science* **265,** 1234–1237
34. Van Kaer, L., Ashton-Rickardt, P. G., Eichelberger, M., Gaczynska, M., Nagashima, K., Rock, K. L., Goldberg, A. L., Doherty, P., and Tonegawa, S. (1994) *Immunity* **1,** 533–541
35. Ashton-Rickardt, P. G., Van Kaer, L., Schumacher, T. N. M., Ploegh, H. L., and Tonegawa, S. (1993) *Cell* **73,** 1041–1049
36. Shepherd, J. C., Schumacker, T. N., Ashton-Rickardt, P. G., Imaeda, S., Ploegh, H. L., Janeway, C. A., Jr., and Tonegawa, S. (1993) *Cell* **74,** 577–584
37. Rock, K. L., Gramm, C., Rothstein, L., Clark, K., Stein, R., Dick, L., Hwang, D., and Goldberg, A. L. (1994) *Cell* **78,** 761–771
38. Cox, A. L., Skipper, J., Chen, Y., Henderson, R. A., Darrow, T. L., Shabanowitz, J., Engelhard, V. H., and Hunt, D. F. (1994) *Science* **264,** 716–719
39. Kozak, M. (1991) *J. Biol. Chem.* **266,** 19867–19870
40. Malarkannan, S., Goth, S., Buchholz, D. R., and Shastri, N. (1995) *J. Immunol.* **154,** 585–598
41. Karttunen, J., Sanderson, S., and Shastri, N. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 6020–6024
42. Sanderson, S., and Shastri, N. (1994) *Int. Immunol.* **6,** 369–376
43. Karttunen, J., and Shastri, N. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 3972–3976
44. Falk, K., Rötzschke, O., and Rammensee, H.-G. (1990) *Nature* **348,** 248–251
45. Rotzschke, O., Falk, K., Stevanovic, S., Jung, G., Walden, P., and Rammensee, H.-G. (1991) *Eur. J. Immunol.* **21,** 2891–2894
46. Falk, K., Rotzschke, O., Faath, S., Goth, S., Graef, I., Shastri, N., and Rammensee, H.-G. (1993) *Cell Immunol.* **150,** 447–452
47. Lapham, C. K., Bacik, I., Yewdell, J. W., Kane, K. P., and Bennink, J. R. (1993) *J. Exp. Med.* **177,** 1633–1641
48. Jackson, M. R., Song, E. S., Yang, Y., and Peterson, P. A. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 12117–12121
49. Bluestone, J. A., Jameson, S., Miller, S., and Dick, R., II (1992) *J. Exp. Med.* **176,** 1757–1761
50. Catipovic, B., Porto, J. D., Mage, M., Johanson, T. E., and Schneck, J. P. (1992) *J. Exp. Med.* **176,** 1611–1618
51. Neefjes, J. J., Momburg, F., and Hammerling, G. J. (1993) *Science* **261,** 769–771
52. Momburg, F., Roelse, J., Howard, J. C., Butcher, G. W., Hammerling, G. J., and Neefjes, J. J. (1994) *Nature* **367,** 648–651
53. Schumacher, T. N. M., Kantesaria, D. V., Heemels, M.-T., Ashton-Rickardt, P. G., Shepherd, J. C., Fruh, K., Yang, Y., Peterson, P. A., Tonegawa, S., and Ploegh, H. L. (1994) *J. Exp. Med.* **179,** 533–540
54. Androlewicz, M. J., and Cresswell, P. (1994) *Immunity* **1,** 7–14
55. Matsumura, M., Saito, Y., Jackson, M. R., Song, E. S., and Peterson, P. A. (1992) *J. Biol. Chem.* **267,** 23589–23595
56. Anderson, K., Cresswell, P., Gammon, M., Hermes, J., Williamson, A., and Zweerink, H. (1991) *J. Exp. Med.* **174,** 489–492
57. Henderson, R. A., Michel, H., Sakaguchi, K., Shabanowitz, J., Appella, E., Hunt, D. F., and Engelhard, V. H. (1992) *Science* **255,** 1264–1266
58. Zweerink, H. J., Gammon, M. C., Utz, U., Sauma, S. Y., Harrer, T., Hawkins, J. C., Johnson, R. P., Sirotina, A., Hermes, J. D., Walker, B. D., and Biddison, W. E. (1993) *J. Immunol.* **150,** 1763–1771
59. Hosken, N. A., and Bevan, M. J. (1990) *Science* **248,** 367–370
60. Rajagopalan, S., and Brenner, M. (1994) *J. Exp. Med.* **180,** 407–412
61. Dick, L. R., Aldrich, C., Jameson, S. C., Moomaw, C. R., Pramanik, B. C., Doyle, C., DeMartino, G. N., Bevan, M. J., Forman, J. M., and Slaughter, C. A. (1994) *J. Immunol.* **152,** 3884–3894
62. Udaka, K., Tsomides, T. J., Walden, P., Fukusen, N., and Eisen, H. N. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 11272–11276
63. Urban, R. G., Chicz, R. M., Lane, W. S., Strominger, J. L., Rehm, A., Kenter, M. J. H., UytdeHaag, F. G. C. M., Ploegh, H., Uchanska-Ziegler, B., and Ziegler, A. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 1534–1538
64. Joyce, S., Kuzushima, K., Kepecs, G., Angeletti, R. H., and Nathenson, S. G. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 4145–4149
65. Eisenlohr, L. C., Bacik, I., Bennink, J. R., Bernstein, K., and Yewdell, J. W. (1992) *Cell* **71,** 963–972
66. Bacik, I., Cox, J. H., Anderson, R., Yewdell, J. W., and Bennink, J. R. (1994) *J. Immunol.* **152,** 381–387