Processing of Major Histocompatibility Class I-restricted Antigens in the Endoplasmic Reticulum

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Summary

We have introduced long precursor peptides directly into the endoplasmic reticulum (ER) of a mutant cell line (T2-D^b) that lacks the ability to transport peptides from the cytosol to the ER in a transporter associated with antigen processing (TAP)–dependent way. This was done by expressing various influenza A–derived peptides containing the naturally processed epitope ASNENMDAM (366–374) preceded by the influenza hemagglutinin ER translocation sequence. Peptides derived from these minigenes that became associated with D^b were isolated and identified by combined reversed phase liquid chromatography and detection by cytotoxic T lymphocytes. Our results establish that NH2-terminal extensions of at least 40 residues can be trimmed from peptides entering the ER, but that proteolysis of larger proteins may be limited.

C TLs recognize peptide epitopes of 8–10 amino acids, bound to polymorphic receptors encoded by the major histocompatibility complex (1–3). The MHC peptide/class I complex is formed in the endoplasmic reticulum (ER)[1] before it translocates to the cell surface. Peptide binding to newly synthesized class I molecules is an integral part of the stable assembly of the MHC molecule itself and may be a prerequisite for transit of the complex to the cell surface (4, 5).

The production of most peptides destined for presentation by class I MHC begins in the cytosol with the limited hydrolysis of antigenic proteins. The proteases involved in this step have not yet been identified, but attention has recently focused on the proteasome — a multicatalytic complex in the cytosol with one ATP-dependent proteolytic activity, specific for ubiquitinated substrates, and three ATP-independent activities (6). The involvement of the former activity in antigen processing has been implied in two experimental systems (7, 8). To date, there is no evidence that proteasomes generate peptides of a size that is optimal for binding to class I. However, recent investigations into the regulation of proteasome specificity by its two MHC-encoded subunits has led to the speculation that proteasomes may generate peptides with COOH termini that are appropriate for binding

to class I (9, 10) and that these may be trimmed in the ER at their NH2 termini, as suggested by Falk et al. (11).

The topological paradox posed by the observations that peptide production and peptide binding to class I occur in two distinct intracellular compartments can be resolved only if there exists a mechanism for translocating peptides across the ER membrane in a signal sequence–independent way (12, 13). Recently, a polymorphic, heterodimeric protein encoded in the MHC class II region has been identified that fulfills this function (14–17). The complex, known as TAP (transporter associated with antigen processing [reviewed in reference 18]), belongs to a family of ATP-binding cassette proteins and has been shown to transport peptides from the cytosolic to the lumenal side of the ER membrane in an ATP-dependent way (19, 20).

The processes by which short peptides are selected for presentation at the cell surface are not fully understood. There is ample evidence for the involvement of the class I molecule itself in selecting peptides, with which it forms the most stable complexes, during the assembly of the heavy chain:β_2 microglobin (β_2m):peptide heterotrimer (11 and reviewed in reference 21). However, the observation that few of the total number of class I–binding peptides that could be generated from any given viral protein are actually selected for use as epitopes suggests that other processes could contribute to epitope selection in vivo. This notion is supported by examples in which different peptide epitopes are presented by the same class I molecule depending on the cellular context in which that class I molecule is expressed (22). Attention has therefore turned to the influence of proteases and the peptide transporters on selecting peptides for class I binding.

Recent evidence has shown that peptides longer than 9 amino acids can be efficiently transported into the ER. The first indication for this came from the demonstration that peptides of up to 11 amino acids, derived from cytosolic and nuclear proteins could be isolated from class I molecules (23). In addition, Udaka et al. (24) have isolated a 16-amino acid peptide from H-2L^d that is derived from the cytosolically synthesized enzyme 2-oxoglutarate dehydrogenase and that is recognized by allospecific CTLs. Recently, direct measurements of the ATP-dependent transport function of the TAP complex have been made (19, 20). These show that long peptides (up to 23 amino acids) are capable of competing against shorter peptides for transport (20). Further indirect evidence for the transport of long peptides by the TAP complex has come from Eisenlohr et al. (25), who have shown that an H-2K^b-restricted epitope could be generated from a 12-amino acid precursor synthesized as a minigene only if (a) an appropriate proteolytic enzyme specificity was coexpressed in the ER and (b) the TAP complex was functional. These results could be explained only if the longer precursor peptide was translocated into the ER by the TAP complex, followed by cleavage to the nonamer.

We demonstrate here that in cells lacking TAP proteins, large fragments of influenza nucleoprotein (NP) translocated into the ER via a hydrophobic signal sequence can be trimmed to the authentic 9-residue epitope recognized by CTLs. At least 40 amino acids between the signal sequence and the epitope can be removed, although larger extensions inhibit trimming. These results establish that an NH$_2$-terminal trimming mechanism does exist in the ER lumen and show that assembly of the resulting short peptide with class I molecules under these conditions can occur in the absence of the TAP proteins.

Materials and Methods

Cells, Antibodies and Peptides. The TAP-defective cell line T2 was transfected with H-2D^b and H-2K^k as previously described (26). Murine L and L-D^b cells were maintained in RPMI 1640 plus 10% FCS (R10) as described previously (1). CTL clone F5 recognizes the D^b-restricted influenza NP epitope ASNENMDAM (corresponding to residues 366–374), as well as longer peptides containing this core sequence (1, 3). HA8 is a CTL clone derived by Gould et al. (27) that recognizes the hemagglutinin (HA)-derived peptide IEGGWTGMI (HA1 residues 354–362) restricted by H-2K^k (27). A polyclonal CTL line recognizing the K^k-restricted influenza NP epitope SDYEGRLI (50–57) was generated from the splenocytes of CBA mice infected with influenza strain E61-13-H17 (H17) and restimulated in vitro with NP50–57 as previously described (28).

The mAb B22.249, specific for the α1 domain of H-2D^b, has been described previously (29). This antibody has been shown to stabilize the interaction between D^b heavy chain and β$_2$m (30, 31) and, when bound to peptide: D^b complexes, slows the rate of dissociation of bound peptides by around 10-fold (32).

Peptides MQIASNENMDAM, QIASNENMDAM, IASNENMDAM, and ASNENMDAM were synthesized manually using fmoc chemistry. Cleavage and deprotection was with TFA. Peptides were purified by ether precipitation followed by reverse phase liquid chromatography (RPLC). Peptides IEGGWTGMI and SDYEGRLI were prepared commercially. All peptides used in this investigation were judged to be >95% pure by HPLC. Determination of peptide concentration was by colorimetric assay (BCA; Pierce, Rockford, IL).

Recombinant Vaccinias. Recombinant vaccinias were made by homologous recombination into the thymidine kinase gene using the shuttle vector psc11.30R.2. This was constructed by blunt-end cloning a synthetic polylinker into the SmaI site of psc11 (33) containing the Kozak sequence, NcoI (containing the ATG codon), SmaI, and StuI sites in two additional reading frames, followed by stop codons in all three reading frames (34). Vaccinias were propagated in tk143 cells and isolated by standard procedures. Virus titers were determined on monolayers of tk143 cells. The recombinant genes introduced into vaccinia for use in this investigation are shown in Fig. 1.

psc11L+M366 and psc11L+M364 were made by first cloning a synthetic minigene coding for the HA-1 leader sequence MKANLLVLLCALAAADA into the unique NcoI site of pscll.30R.2 in such a way as to preserve a unique NcoI site immediately 3' of the inserted leader sequence. The orientation and sequence of the new plasmid (psc11L+) were confirmed by dideoxy sequencing. Oligos encoding either MASNENMDAM or MQIASNENMDAM were constructed with a 5' NcoI site (encoding Met at position 1) and a BamHI site at the 3' end. These were cloned into NcoI/BglII-cut psc11L+. psc11L+NP was made in a similar way, by ligating the NcoI–SalI fragment of ptac85-NP (7) containing the full-length influenza NP with NcoI/SmaI-cut psc11L+, after creating a blunt end at the 3' end of NP. psc11L+IMP was made by cloning a synthetic minigene coding for MKANLLVLLCALAAADAMNAT, which contains the HA leader sequence, into the EcoRI/PvuII-cleaved fragment of ptac85-NP (7). The EcoRI–SalI fragment of this plasmid containing the leader plus residues 328–498 of NP was then blunt-end cloned into SmaI-cut psc11. Generation of 365–379 vacc, IMP vacc, and NP vacc have been described previously (7, 35).

CTL Assays. 4-h CTL assays were performed using a standard protocol. When sensitization by recombinant vaccinias was tested, 10^6 target cells were incubated with 5 × 10^6 PFU of virus and 20 μCi of ^{51}Cr (as sodium chromate) for 1 h at 37°C. The cell pellet was then washed twice with R10, resuspended in 200 μl of the same, and incubated for an additional 2.5–3 h at 37°C. After three washes with R10, cells were dispensed at 10,000 cells per well for use in the assay. For influenza infection, targets were washed three times in PBS before infection with 200 μl of allantoic fluid containing influenza E61-13-H17 virus in the presence of ^{51}Cr. When peptides were tested, they were added to target and effector cells and were present throughout the CTL assay. When pharmacological interference with processing was tested, target cells were labeled and then preincubated with either 0.5 μg of brefeldin A (BFA) or 80 μM chloroquine for 20 min at room temperature before the addition of virus. They were then washed with R10 made either 1 μM BFA or 60 μM chloroquine before infection in the same. CTL assays were performed in the presence of 1 μM BFA or 5 μM chloroquine.

Large-Scale Infection of Cells and Immunoprecipitation of D^b from Infected Cells. For peptide elution experiments, 10^9 T2-D^b or L-D^b cells were harvested and resuspended in RPMI 1640 supplemented with glutamine at a final concentration of 10^8 cells per ml, in the presence of 5 × 10^9 PFU of the appropriate purified vaccinia. After 2 h at 37°C with continual rolling, the cell pellet was washed once with R10, resuspended in 50 ml of the same, and incubated on a roller platform for 4–6 h at 37°C. (Expression of vaccinia-encoded genes was confirmed by monitoring β-galactosidase ex-
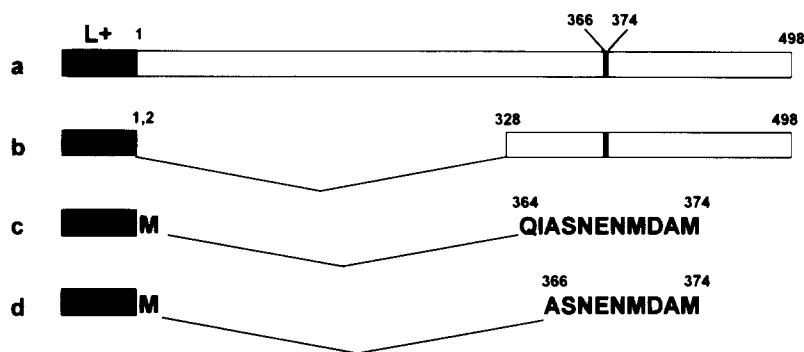
Figure 1. Constructs used in this investigation. The vaccinia shuttle vector psc11.30R.2 containing L+NP (a), L+IMP (b), L+M364-374 (c), and L+M366-374 (d) were used to make recombinant vaccinias as described in Materials and Methods.

pression in aliquots of 2 × 10⁵ cells that were incubated with 6 mg/ml X-Gal for 1 h at 37°C after being lysed in 100 μl of double-distilled H₂O. The supernatant was monitored for absorbance at 405 nm. Typically, infected cells reached a plateau of expression at OD 1.0–2.0 after around 2 h (wild-type infected cells gave an OD of 0.2). The cell pellet was then harvested, washed twice in ice-cold Tris-buffered saline (TBS), and immediately lysed by the addition of 5 ml of ice-cold TBS containing 1% NP-40, protease inhibitors (5 mM EDTA, PMSF, iodoacetamide, 50 μg/ml antipain, 2 μg/ml aprotinin, 40 μg/ml bestatin, 100 μg/ml chymostatin, 10 μg/ml E-64, 0.5 μg/ml leupeptin, 0.7 μg/ml pepstatin, and Nα-p-tosyl-L-chloromethyl ketone [TLCK]), and 1 mg of purified B22.249. After 2 h at 4°C with continual agitation, the lysate was centrifuged at 15,000 rpm, and the cleared supernatant was harvested. 2 ml of a 10% suspension of protein A-Sepharose 4B in TBS, 1% NP-40 was added, and incubation was continued for an additional 1 h at 4°C. The immunoprecipitate was then washed twice in 10 ml of TBS, 1% NP-40 and four times in 10 mM ammonium acetate, pH 7.5 at 4°C.

Extraction and Analysis of Peptides Bound to Dᵇ. Washed precipitates were incubated for 5 min at room temperature with 0.1% TFA or 10% acetic acid in water. Protein A-Sepharose beads were removed by filtration through a 0.45-μm membrane, and low molecular weight material (<10,000) was isolated by membrane filtration chromatography (Centricon C10; Amicon Corp., Beverly, MA). Samples of 1–2 ml recovered in this way were applied to a C8 microbore RPLC (Applied Biosystems, Inc., Foster City, CA) column without further processing and fractionated by gradient elution (2–50% acetonitrile in 0.07% TFA at a rate of 1% per min followed by 50–90% at a rate of 2% per min) at a flow rate of 0.2 ml/min. 1-min fractions were collected and immediately dried under vacuum. 200 μl of RPMI 1640 was added to each tube, and 50 μl of the redissolved fraction was tested in duplicate for its ability to sensitize labeled T2-Dᵇ for lysis by clone F5. In addition, each fraction was tested in duplicate for its toxicity to target cells in the absence of specific CTLs.

Immunoprecipitation of NP. 5 × 10⁶ T2-Dᵇ cells were infected for 1 h with 1.5 × 10⁷ PFU of L+NP-vacc or L-NP-vacc, in 100 μl of methionine-free RPMI supplemented with 10% FCS. 50 μCi of [³⁵S]methionine was then added to each aliquot of cells, and metabolic labeling was allowed to proceed for 30 min at 37°C. Cells were then lysed in 0.5 ml of cold Tris buffer, pH 7.4, containing 0.5% NP-40 and 0.5% Mega-9 (Sigma Immunochemicals, St. Louis, MO), and the lysates were precleared overnight with fixed *Staphylococcus aureus.* Immunoprecipitation of NP was performed the following day using the anti–influenza A NP mAb 14-7-18 as described previously (12). Endoglycosidase H digestion of immunoprecipitates was performed by resuspending the immuno-

precipitate in 30 μl of 50 mM sodium citrate buffer, pH 5.5, containing 0.2% SDS, heating to 95°C for 5 min, and adding 2 U of endoglycodidase H after cooling to 37°C. Digestion was performed at 37°C for 18 h before resolving the immunoprecipitates on a 10% gel.

Results

NH₂-Terminal Trimming Occurs in the ER of TAP-deficient Cells. The immunodominant Dᵇ-restricted epitope of influenza A virus is the nonamer ASNENMDAM, corresponding to residues 366–374 of the NP (1, 3). This peptide has been isolated from Dᵇ molecules purified from lysates of influenza-infected cells (3). We have shown previously that 366–374 binds to Dᵇ with a half-life of >200 h at 4°C and that peptides extended at the NH₂ terminus also bind but with an intermediate rate of dissociation, around 20 h at 4°C for the decamer Y366-374. By contrast, peptides extended at the COOH terminus dissociated 10-fold faster than those extended at the NH₂ terminus (32). These results were consistent with the proposal by Rotszchke et al. (3) that peptides extended at the NH₂ terminus may bind to class I molecules in the ER, albeit with low affinity, before trimming.

To determine whether peptides longer than the optimal nonameric epitope can be trimmed by ER enzymes to produce the optimal peptide, we constructed recombinant vaccinia viruses containing minigenes encoding either the 10–amino acid peptide MASNENMDAM or the 12–amino acid peptide MQIASNENMDAM, tagged with the signal sequence derived from influenza A/PR/8/34 HA (called L+M366-vacc and L+M364-vacc; see Fig. 1). Addition of a hydrophobic signal sequence has been shown to be an efficient way of by-passing the TAP defect in T2 (36). Fig. 2 *b* shows that these vaccinias were as efficient in sensitizing L-Dᵇ target cells for lysis by the CTL clone F5 as the minigenes synthesized without a leader sequence (L-M366-vacc and L-365-379-vacc). However, only those peptides expressed with the HA leader sequence were able to sensitize T2-Dᵇ cells for lysis by F5 (Fig. 2 *d*), indicating that efficient delivery of the peptide products to Dᵇ molecules was dependent on the presence of the ER translocation sequence in a cell line that lacks a functional TAP complex. This established that the fragments must have been translocated with their NH₂-terminal extensions intact.

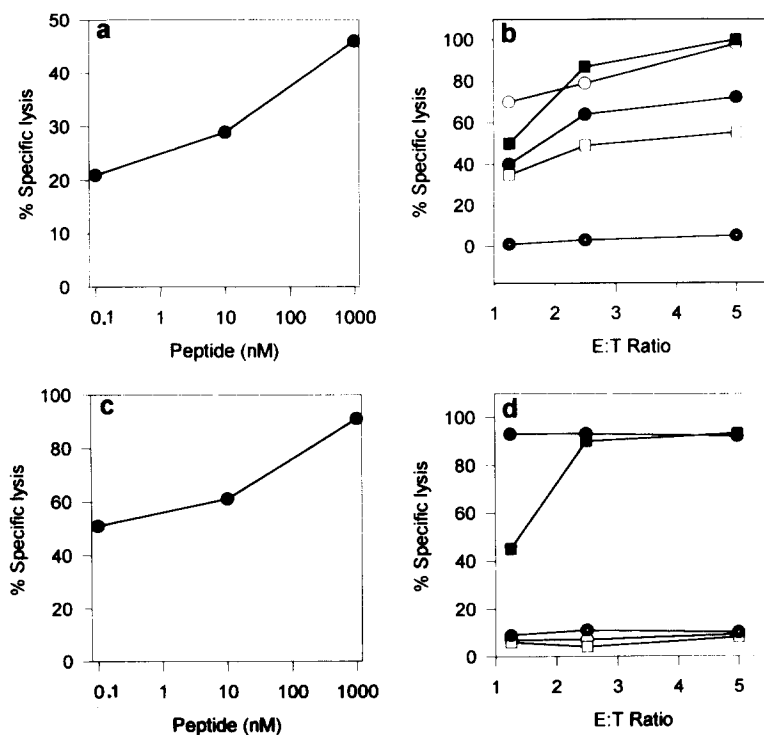We next asked whether the predominant epitope recog-

Figure 2. Sensitization of target cells with L+peptide-vacc. The ability of clone F5 to lyse either L-D^b (a and b) or T2-D^b (c and d) cells was tested after preincubation with either synthetic peptide 366–374 (a and c) or recombinant vaccinias (b and d) encoding L+M366–374 (●), M366–374 (O), L+M364–374 (■), M365–379 (□), or HIV-pol (●). The E/T ratio in a and c was 2:1.

nized by F5 under these conditions corresponded to the sequence encoded by the minigene, or whether further processing of the NH$_2$-terminal extended peptides had occurred in the ER. We isolated peptides associated with D^b after infection with the appropriate recombinant vaccinia and identified the predominant epitope according to its characteristic retention time upon RPLC fractionation. First, each potential product, MQIASNENMDAM, QIASNENMDAM, IASNENMDAM, and ASNENMDAM, was synthesized and was shown to give a characteristic retention time when analyzed by RPLC. The relative ability of each peptide to sensitize T2-D^b in a CTL assay was also determined. Fig. 3 a shows that the naturally processed peptide 366–374 sensitized T2-D^b cells to half-maximal lysis at a concentration of 1 pM. To our surprise, M364–374 was as effective, followed by 365–374 and 364–374, which was over 10,000-fold less potent. These results indicate that successful detection of both M364–374 and 366–374 could be achieved by the recovery of each peptide from infected cells in the femtomolar range. The method for purifying D^b-bound peptides was chosen to ensure that loss of peptides with a short half-life of dissociation was minimal (see Materials and Methods).

Fig. 3 c and d show that after infection with either L+M366–374 or L+M364–374, only the nonamer AS-NENMDAM could be recovered from D^b, indicating that the longer precursors, introduced directly into the ER of T2, had been trimmed to the optimal epitope. No intermediate sized peptides were identified. The control infection with wild-type vaccinia WR (Fig. 3 b) did not give rise to any peptide capable of sensitizing T2-D^b cells.

ER Proteases Are Capable of Generating Optimal Epitopes

from Large Polypeptide Precursors. To see whether ER proteolysis was limited to the trimming of a small number of amino acids from the NH$_2$ termini of long precursor peptides, or whether the same optimal epitope could be derived from a much larger precursor, recombinant vaccinias were constructed that encoded a 170–amino acid COOH-terminal fragment of NP (residues 328–498, referred to as IMP) with and without the HA leader sequence (termed L+IMP and L-IMP, respectively, see Fig. 1). We were unable to detect the presence of this fragment after infection of T2-D^b cells with L+IMP by immunoprecipitation or Western blotting. The IMP fragment expressed without the leader sequence can be detected by immunoprecipitation and has been shown to be rapidly degraded in the cytosol (7). Lack of detection of L+IMP most probably therefore reflects its very short half-life when expressed with the ER translocation sequence. Other proteins that have been inappropriately expressed in the ER after experimental manipulation have been shown to have half-lives on the order of minutes, even though they are relatively stable when expressed in their native compartment (37). Despite the lack of biochemical evidence for expression, Fig. 4 shows that sensitization of L-D^b cells by L+IMP was as efficient as that by L-IMP, indicating that IMP was efficiently expressed by the L+minigene construct. Furthermore, sensitization of T2-D^b cells by L+IMP-vacc was efficient and was dependent on the presence of the leader sequence.

Isolation of peptides associated with D^b from L+IMP–infected T2-D^b cells was performed to determine the nature of the epitope derived from this polypeptide. Fig. 4 shows that a major species corresponding to ASNENMDAM was identified. Thus, again, the major product of intracellular
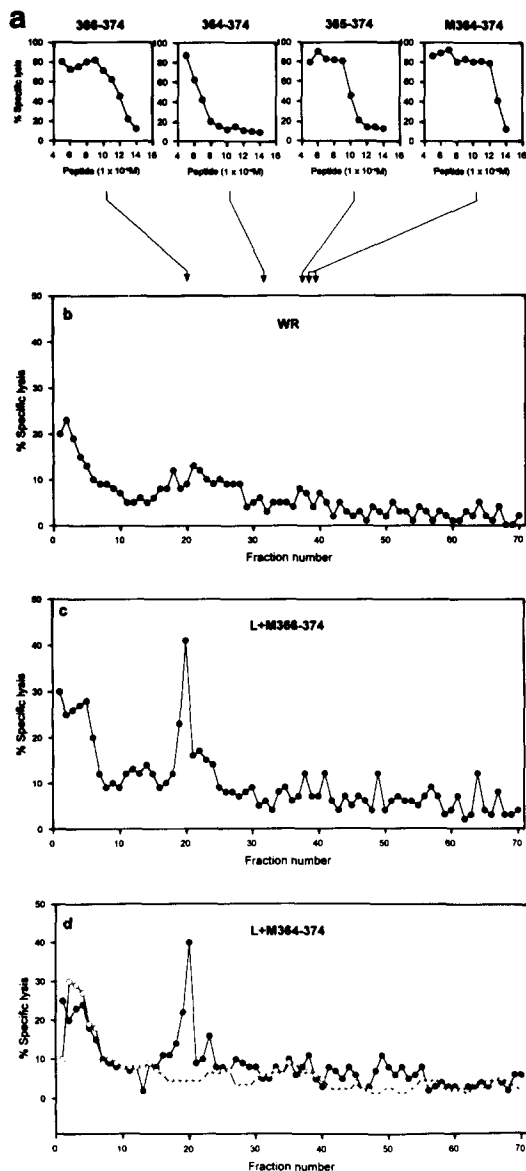
Figure 3. Characterization of peptides associated with D[b] isolated from recombinant vaccinia–infected T2-D[b] cells. (a) Titration curves for 366–374, 365–374, 364–374, and M366–374 and their characteristic retention times on RPLC. Detection of peptides by clone F5 (●) was tested after fractionation of peptides associated with D[b] from T2-D[b] cells infected with wild-type vaccinia (b), L+M366–374 vacc (c), and L+M364–374 vacc (d). In addition, the nonspecific toxicity of each fraction was tested (O).

proteolysis was the nonamer corresponding to the optimal binding peptide. These results show that proteolysis in the ER can give rise to an optimal CTL epitope contained within a 170–amino acid polypeptide, in which the epitope is ∼40 residues from the NH₂ terminus.

In addition, fraction 58 sensitized T2-D[b] cells for lysis in a CTL-dependent way, though this was not reproducible when the experiment was repeated. In the experiment shown in Fig. 4, we were unable to determine the origin of this activity, though the possibility that it represented an intermediate of proteolysis cannot be ruled out.

Trimming Does Not Occur Extracellularly. Since the precursor peptides expressed with the ER translocation signal were all introduced directly into the secretory pathway, it was possible that the trimming we observed could have occurred in the extracellular space during infection, followed by binding to D[b] at the cell surface. To rule out this possibility, we coincubated ⁵¹Cr-labeled T2-D[b] cells with unlabeled T2 cells that had been infected with L+vacc. Coincubation was performed for 4–6 h under the same conditions as those used for infection before the isolation of D[b]. This mixture of cells was then exposed to clone F5 in a 4-h CTL assay. The labeled, uninfected T2-D[b] cells were not sensitized for lysis after coincubation with infected T2 cells even when the latter were present at a 10-fold excess. In the same experiment, T2-D[b] cells were sensitized for lysis by infection under the same conditions and by incubation with added peptides down to a concentration of 0.1 pM (data not shown).

Another explanation for the observed trimming of precursor peptides was that they were degraded in vitro after lysing the infected cells, before immunoprecipitation. We addressed this possibility by lysing 10⁹ T2-D[b] cells in the presence of 2 μM crude M364–374. D[b] molecules were immunopurified. After washing the immunoprecipitate and eluting bound peptides, the peptide mixture was fractionated by RPLC, and each fraction was tested for its ability to sensitize T2-D[b] cells for lysis by clone F5. Fig. 5 shows that the predominant peak of CTL activity resided in fractions coeluting with RPLC-purified M364–374. A small peak of activity was also seen in fraction 20, which almost certainly represents the presence of some 366–374. (This was confirmed by performing the same experiment with 2 μM 366–374 in the lysate, in which a large peak of activity was recovered from immunopurified D[b] in fractions spanning 20; see Fig. 5.) Some of this material was already present in the M366–374 preparation, since the same peak of activity was detected when the peptide was fractionated by RPLC and each fraction was tested (data not shown). Inclusion of protease inhibitors in the lysis buffer was therefore sufficient to prevent the complete degradation of M364–374 during the peptide isolation procedure. Taken together, these results indicate that the recovery of 366–374 from L+M364–374–infected cells was a consequence of intracellular processing before lysis.

Intracellular Processing of L+ Constructs Occurs in a BFA-sensitive, Chloroquine-insensitive Compartment. BFA causes a redistribution of intracellular compartments such that the medial and cis-Golgi cisternae collapse into the ER, whereas the trans-Golgi network fuses with the recycling endosomal system (38). It therefore blocks class I–restricted presentation of intracellular antigens by inhibiting the egress of newly formed class I:peptide complexes from the ER to the trans-Golgi network (39, 40). We tested the ability of BFA to interfere with the processing and presentation of vaccinia-encoded L+M364–374 and L+IMP. Fig. 6 b shows that 1 μM BFA was sufficient to block presentation of these polypeptides by T2-D[b] cells. Inhibition of presentation of both the L+ and L-polypeptides in L-D[b] cells, which has a functional TAP complex, was also observed (Fig. 6 a).
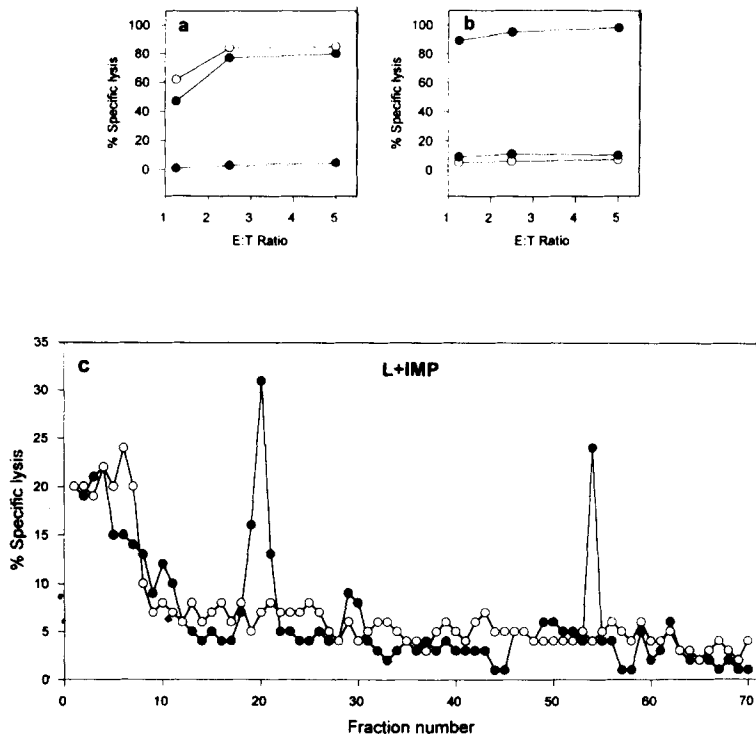
Figure 4. Sensitization of target cells with L+IMP and elution of peptides from L+IMP-infected T2-Dᵇ cells. The ability of clone F5 to lyse either L-Dᵇ (a) or T2-Dᵇ (b) cells was tested after infection with recombinant vaccinias encoding L+IMP (●), IMP (O), or HIV-pol (◉). (c) Peptides associated with Dᵇ from T2-Dᵇ cells infected with L+IMP were fractionated and tested for their ability to sensitize clone F5 (●) or to lyse T2-Dᵇ cells nonspecifically (O).

Leader-dependent, intracellular processing of antigens for presentation with MHC class II has been described previously (41, 42), and has been shown to occur in low pH compartments, by the ability of cloroquine to inhibit antigen processing. Although the requirement for newly synthesized MHC molecules was not demonstrated by these investigations, we felt that it was necessary to investigate the role of low pH compartments in generating epitopes from our L+constructs. Fig. 6 shows that chloroquine had no effect on the processing and presentation of L+M364 and L+IMP in T2-Dᵇ cells (or in the control cell line L-Dᵇ).

Taken together, these results are consistent with the trimming of precursor polypeptides and the association of trimmed products with newly synthesized class I molecules occurring

in a pre-trans-Golgi compartment and most likely the lumen of the ER.

CTL Epitopes Are Not Generated from the ER Degradation of Full-Length Proteins. It is unclear whether CTL epitopes
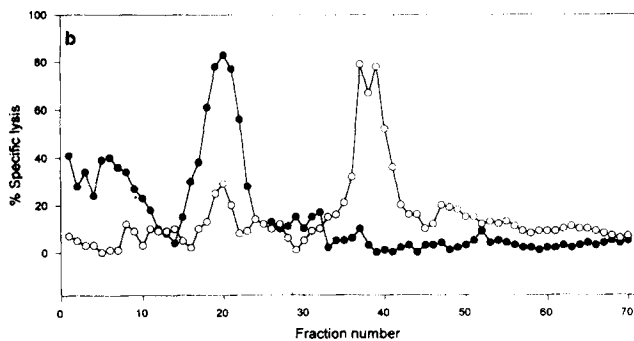

Figure 5. Trimming is not due to extracellular processing. Little in vitro degradation of M366-374 occurs after cell lysis. T2-Dᵇ cells were lysed in the presence of 366-374 (●) or M364-374 (O), and peptides bound to Dᵇ were eluted and fractionated by RPLC.
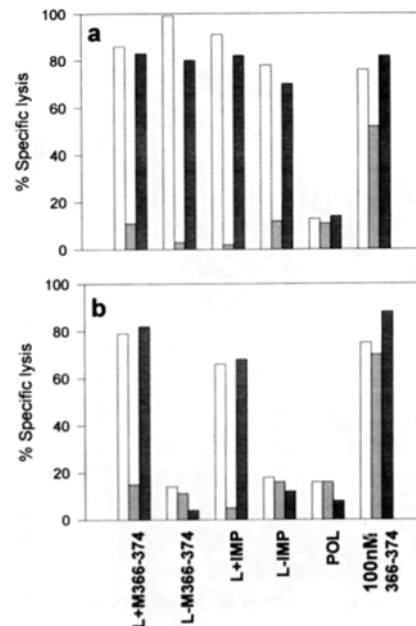

Figure 6. The ability of L-Dᵇ (a) and T2-Dᵇ (b) cells to process L+M366-374 vacc, L+M364-374 vacc, and L+IMP vacc and to present synthetic 366-374 to clone F5 in the absence of drugs (open bars) or in the presence of BFA (dashed bars) or chloroquine (hatched bars).

can be generated in the ER from glycoproteins. Indeed, the observation that CTL epitopes appear to be derived mainly from nonglycoproteins and the demonstration that peptides derived from glycoproteins and secreted proteins occur rarely in eluates from purified class I molecules (see reference 21 for a summary) suggest that glycoproteins are protected from the action of ER proteases.

We have investigated the role of ER proteases in the processing of glycoproteins in two ways. First, the processing of influenza A HA was investigated in T2-K^k. Fig. 7 shows that the HA epitope IEGGWTGMI (corresponding to residues 354–362 of HA1), recognized by the K^k-restricted clone HA8 (27), was not generated in T2-K^k cells when the antigen, was delivered after influenza (PR8) infection or infection with recombinant vaccinias encoding full-length HA (1–565) or a truncated HA (1–413) (27), even though L-D^b cells were sensitized for lysis by all three. Thus, HA and the truncated fragment did not appear to be susceptible to ER degradation in the same way as L+IMP.

In a second approach, we constructed a recombinant vaccinia encoding full-length influenza NP, tagged with the HA leader sequence (L+NP-vacc). Infection of T2-D^b cells with this vaccinia and immunoprecipitation of NP from a pulse-labeled lysate indicated that the majority of NP migrated more slowly than L-NP when analyzed by SDS-PAGE, and was sensitive to endoglycosidase H (Fig. 8). These results are consistent with the cotranslational transport of newly synthesized NP into the ER and the glycosylation of cryptic sequons (NAT and NDT) at positions 21 and 144.
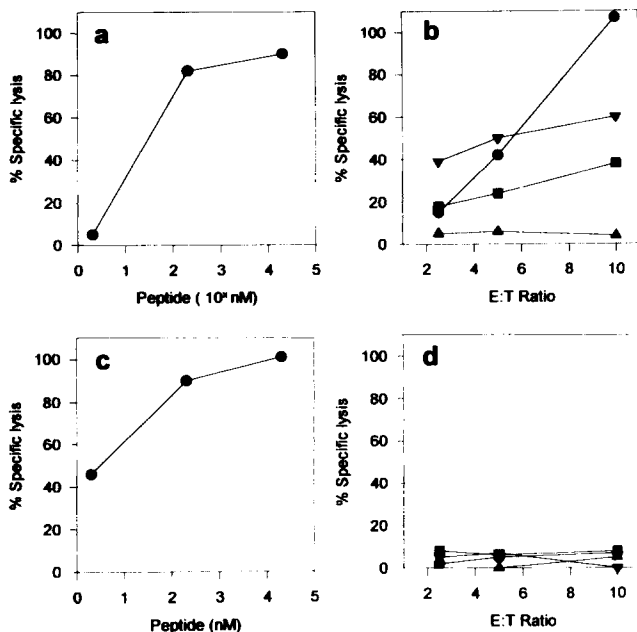

Figure 8. Detection of NP in recombinant vaccinia-infected T2-D^b cells. T2-D^b cells were infected with L+NP-vacc (lanes a and b) or L-NP-vacc (lanes c and d), and NP was detected 1.5 h after infection by immunoprecipitation. Immunoprecipitates were either treated with endoglycosidase H (lanes b and d) or mock digested (lanes a and c).

The ability of ER proteases to generate two well-characterized CTL epitopes from L+NP was then tested. Fig. 9 shows that neither the K^k (SDYEGRLI, 50–57 [43]) nor the D^b (366–374) epitope was produced after infection. The CTLs used in this assay (F5 and a polyclonal anti-SDYEGRLI line) could nevertheless recognize T2 transfectants when pulsed with as little as 0.5 nM of the relevant peptide (Fig. 9 e).

Figure 7. Sensitization of target cells with endogenous influenza HA for lysis by clone HA8. L (a and b) and T2-K^k (c and d) cells were sensitive to lysis by added peptide 354–362 (a and c) and were tested for their ability to process and present this peptide after infection with influenza A PR8 (▼), HA vacc (●), KG26 vacc encoding the HA truncation 1–413 (■), and HIV-pol vacc (▲).
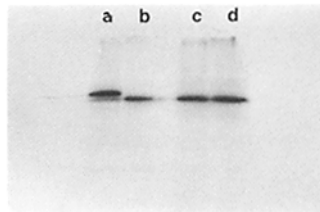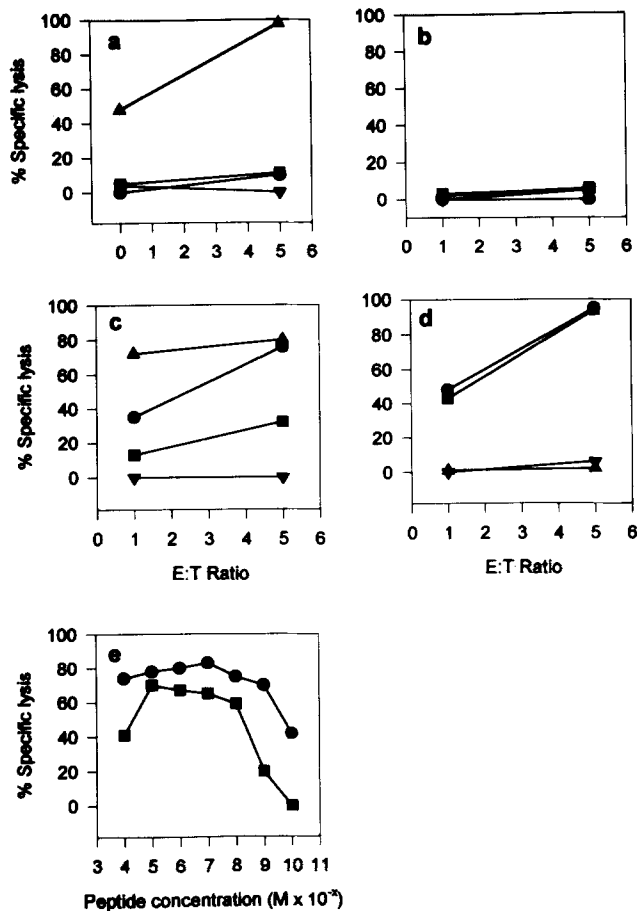
Figure 9. Sensitization of target cells with L+NP. T2-D^b (a), T2-K^k (b), and TAP-competent L-D^b cells, which express both D^b and K^k (c and d), were tested for their ability to present peptide antigen to clone F5 (a and c) and a K^k-restricted polyclonal CTL line raised against NP50–57 (SDYEGRLI, b and d) after infection with recombinant vaccinias encoding L+NP (●), L-NP (■), L+IMP (▲), or HIV-pol (▼). (e) In addition, the sensitivity of each of these CTLs (assayed at an E:T ratio of 2:1 for F5- and 10:1 for the K^k-restricted CTL line) was tested on the appropriate T2 transfectant pulsed with synthetic peptides ASNENMDAM (●) and SDYEGRLI (■).

Discussion

Our results show that proteases in the ER (or other pre-Golgi compartments) can trim a precursor to a short peptide epitope that is optimal for presentation at the cell surface, as postulated by Falk et al. and Rotzschke et al. (3, 11).

These results raise the possibility that the enzymatic processing of intracellular antigens could continue in the same intracellular compartment as that in which peptide binding to MHC class I molecules occurs. The natural substrates for ER proteases could therefore be longer peptides, generated in the cytosol, which are then delivered to the ER lumen by the TAP complex.

Recent work has demonstrated a physical association between class I molecules and the TAP complex in the ER (44, 45). The results of this study show that appropriate trimming and loading of peptides into class I molecules and their presentation at the cell surface can occur in the absence of the TAP complex, implying that contact of class I with TAP molecules is not essential for presentation of antigen.

Peptides encoded by the minigenes L+M364–374 and L+M366–374 (being only 23–26 amino acids) are unlikely to be synthesized on rough ER and are more likely to be posttranslationally translocated in a leader-dependent way (46). By studying the trimming of NH_2-terminal extended peptides only, we were able to rule out any effect of cytosolic proteases, to which the gene products were undoubtedly exposed, since trimming of the NH_2 terminus would destroy the signal sequence and COOH-terminal trimming would lead to peptides that eluted with unique retention times when analyzed by RPLC. Having also ruled out artifacts such as extracellular proteolysis in vitro and demonstrated that the leader-dependent generation of CTL epitopes is BFA sensitive and cloroquine insensitive, it is most likely that the trimming we have observed occurred in a pre-Golgi compartment of the infected cells.

It is therefore possible to envisage a situation in which cytosolic proteases generate fairly large peptide fragments from denatured proteins in the cytosol, which are then transported into the lumen of the ER by the TAP heterodimer. Enzymes in the ER could then trim longer precursor peptides in order to optimize the NH_2 termini for class I binding. The possibility that COOH-terminal trimming can occur in the ER cannot be ruled out, although experiments by Eisenlohr et al. (25) have implied that COOH-terminal trimming of a particular K^d-restricted epitope was not detectable.

We have also found that the 170–amino acid IMP fragment of NP could be processed in the ER via a BFA-sensitive, chloroquine-insensitive pathway and also gave rise to the optimal nonameric epitope NP366–374. This result established that up to 40 amino acids can be removed from between the signal sequence and the epitope. In addition to the removal of 40 amino acids from the NH_2 terminus, 120 amino acids must also be removed from the COOH terminus for this epitope to be produced. The L+IMP construct, being 170 amino acids, is more likely to be cotranslationally transported into the ER than its shorter counterparts (46). It is possible, therefore, that proteases that are normally present in the ER

are capable of degrading IMP in such a way as to reveal the correct COOH-terminal amino acid (Met-374) of the D^b-restricted epitope, as well as being able to trim 40 amino acids from the NH_2 terminus. However, it is also possible that some newly synthesized IMP is exposed to cytosolic proteases, which trim the COOH terminus before transport into the ER.

The ability of ER proteases to degrade full-length NP expressed with a leader sequence revealed that this construct was a poor substrate, despite the synthesis of easily detectable amounts of the L+NP construct. The loss of presentation could be due to an increase in the stability of NP when expressed as a full-length protein. NP might be stabilized in the ER as a result of conformational factors, or as a result of its glycosylation. Our results with influenza HA suggest that the latter may be the more likely, since neither the full-length HA, nor a truncated polypeptide (which would not be expected to adopt the same tertiary structure) gave rise to a K^k-restricted epitope in T2-K^k cells. Both these polypeptides contain glycosylation sites.

These results address the general issue of glycoprotein degradation in the ER and the ability of these degradation products to assemble with class I molecules. Glycoprotein-derived CTL epitopes have been described, but the role of ER proteases in their generation is unclear. Townsend et al. (13) have shown that a K^k-restricted HA epitope can be efficiently generated in a leader-independent way, suggesting that a fraction of this glycoprotein is released from ribosomes into the cytoplasm and exposed to cytosolic proteases. Indeed, in this study, we have shown that the same epitope cannot be generated in the ER, indicating that HA may be processed exclusively in the cytosol. The same may be true of other glycoprotein-derived CTL epitopes. Similar to our observations with influenza HA, measles F protein cannot be presented to B27-restricted CTLs by infected T2-B27, which lacks a functional TAP complex, even though it is readily presented by TAP-competent cells (47). Nevertheless, Hammond et al. (48) have recently demonstrated the ability of T2 cells to process HIV-1 gp120 in a leader-dependent way, giving rise to an HLA-A3–restricted epitope, suggesting that this peptide was generated by enzymes in the ER. This may be a relatively infrequent occurrence and would explain why the majority of CTL epitopes described to date appear to be derived from proteins that do not enter the secretory pathway; it is supported by our observation that neither of the NP epitopes we studied could be generated in the ER. The implication is therefore that glycoproteins may be sheltered from the class I–associated processing pathway by their limited exposure to cytosolic proteases and by their relative resistance to proteolysis in the ER.

Very little is known about ER resident proteases. The best studied to date is leader peptidase, which cleaves the ER translocation signal sequence from type 1 glycoproteins and has a well-defined specificity, cleaving after A, G, S, T, or C, in the general motif (A/G/S/T/C/L/V/I)X(A/G/S/T/C) (46). Peptides derived from leader sequences, generated by the action of leader peptidase have been identified bound to HLA-

A2 in normal and TAP-defective cell lines (49). However, the specificity of this enzyme makes it unlikely that it is responsible for the NH$_2$-terminal trimming observed in our experiments, since the sequences immediately preceding 366–374 in either L+M366–374 (MQI) or L+M364–374 (DAM) do not contain a motif susceptible to recognition by leader peptidase. The involvement of leader peptidase in trimming the NH$_2$ terminus of L+IMP and L+NP is even less likely.

Selective, rapid degradation of incompletely assembled multichain glycoproteins has been described for TCR α and β and CD3δ (50, 51). These polypeptides contain structural elements in their transmembrane domains that predispose them to proteolysis in the ER if they are not masked by multisubunit assembly. Although none of our L+ constructs contain this motif, the enzymes responsible for their degradation may be the same as those recruited for the degradation of TCR chains.

Recently, Wileman et al. (52) have shown that targetted degradation of TCR chains in the ER can be inhibited by inhibitors of cysteine proteases and not by specific inhibitors of trypsinlike serine proteases. The degradation of many other incompletely assembled or misfolded proteins in the ER has been investigated, and the involvement of calcium-dependent proteases (53) and TLCK-inhibitable serine proteases (54) has been implicated in the proteolysis of two of these. It therefore appears that all classes of protease have been identified in association with the ER. It remains to be seen which of these activities, if any, are responsible for generating peptides suitable for assembly with class I MHC. In addition, the possibility that the ER is subdivided into functionally specialized compartments (55), with distinct regions for specialized proteolysis, is intriguing.

References

1. Townsend, A.R., J. Rothbard, F.M. Gotch, G. Bahadur, D. Wraith, and A.J. McMichael. 1986. The epitopes of influenza nucleoprotein recognized by cytotoxic T lymphocytes can be defined with short synthetic peptides. *Cell.* 44:959–968.
2. van Bleek, G.M., and S.G. Nathenson. 1990. Isolation of an endogenously processed immunodominant viral peptide from the class I H-2Kb molecule. *Nature (Lond.).* 348:213–216.
3. Rotzschke, O., K. Falk, K. Deres, H. Schild, M. Norda, J. Metzger, G. Jung, and H.G. Rammensee. 1990. Isolation and analysis of naturally processed viral peptides as recognized by cytotoxic T cells. *Nature (Lond.).* 348:252–254.
4. Townsend, A., C. Ohlen, J. Bastin, H.G. Ljunggren, L. Foster, and K. Karre. 1989. Association of class I major histocompatibility heavy and light chains induced by viral peptides. *Nature (Lond.).* 340:443–448.
5. Lapham, C.K., I. Bacik, J.W. Yewdell, K.P. Kane, and J.R. Bennink. 1993. Class I molecules retained in the endoplasmic reticulum bind antigenic peptides. *J. Exp. Med.* 177:1633–1641.
6. Goldberg, A.L., and K.L. Rock. 1992. Proteolysis, proteasomes and antigen presentation. *Nature (Lond.).* 357:375–379.
7. Townsend, A., J. Bastin, K. Gould, G. Brownlee, M. Andrew, B. Coupar, D. Boyle, S. Chan, and G. Smith. 1988. Defective presentation to class I–restricted cytotoxic T lymphocytes in vaccinia-infected cells is overcome by enhanced degradation of antigen. *J. Exp. Med.* 168:1211–1224.
8. Michalek, M.T., E.P. Grant, C. Gramm, A.L. Goldberg, and

K.L. Rock. 1993. A role for the ubiquitin-dependent proteolytic pathway in MHC class I-restricted antigen presentation. *Nature (Lond.).* 363:552–554.
9. Driscoll, J., M.G. Brown, D. Finley, and J.J. Monaco. 1993. MHC-linked LMP gene products specifically alter peptidase activities of the proteasome. *Nature (Lond.).* 365:262–264.
10. Gaczynska, M., K.L. Rock, and A.L. Goldberg. 1993. Gamma-interferon and expression of MHC genes regulate peptide hydrolysis by proteasomes. *Nature (Lond.).* 365:264–267.
11. Falk, K., O. Rotzschke, and H.G. Rammensee. 1990. Cellular peptide composition governed by major histocompatibility complex class I molecules. *Nature (Lond.).* 348:248–251.
12. Townsend, A.R., F.M. Gotch, and J. Davey. 1985. Cytotoxic T cells recognize fragments of the influenza nucleoprotein. *Cell.* 42:457–467.
13. Townsend, A.R., J. Bastin, K. Gould, and G.G. Brownlee. 1986. Cytotoxic T lymphocytes recognize influenza haemagglutinin that lacks a signal sequence. *Nature (Lond.).* 324:575–577.
14. Monaco, J.J., S. Cho, and M. Attaya. 1990. Transport protein genes in the murine MHC: possible implications for antigen processing. *Science (Wash. DC).* 250:1723–1726.
15. Spies, T., M. Bresnahan, S. Bahram, D. Arnold, G. Blanck, E. Mellins, D. Pious, and R. DeMars. 1990. A gene in the human major histocompatibility complex class II region controlling the class I antigen presentation pathway. *Nature (Lond.).*

348:744–747.

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

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

18. Townsend, A., and J. Trowsdale. 1993. The transporters associated with antigen presentation. Semin. Cell Biol. 4:53–61.

19. Neefjes, J.J., F. Momburg, and G.J. Hammerling. 1993. Selective and ATP-dependent peptide translocation of peptides by the MHC-encoded transporter. Science (Wash. DC). 261:769–771.

20. Shepherd, J.C., T.N. Schumacher, P.G. Ashton Rickardt, S. Imaeda, H.L. Ploegh, C.A.J. Janeway, and S. Tonegawa. 1993. TAP1-dependent peptide translocation in vitro is ATP dependent and peptide selective. Cell. 74:577–584.

21. Elliott, T., M. Smith, P. Driscoll, and A. McMichael. 1994. Peptide selection by class I molecules of the major histocompatibility complex. Curr. Biol. 3:854–866.

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

23. Guo, H.C., T.S. Jardetzky, T.P. Garrett, W.S. Lane, J.L. Strominger, and D.C. Wiley. 1992. Different length peptides bind to HLA-Aw68 similarly at their ends but bulge out in the middle. Nature (Lond.). 360:364–366.

24. Udaka, K., T.J. Tsomides, P. Walden, N. Fukusen, and H.N. Eisen. 1993. A ubiquitous protein is the source of naturally occurring peptides that are recognized by a CD8+ T-cell clone. Proc. Natl. Acad. Sci. USA. 90:11272–11276.

25. Eisenlohr, L.C., I. Bacik, J.R. Bennink, K. Bernstein, and J.W. Yewdell. 1992. Expression of a membrane protease enhances presentation of endogenous antigens to MHC class I-restricted T lymphocytes. Cell. 71:963–972.

26. Alexander, J., J.A. Payne, R. Murray, J.A. Frelinger, and P. Cresswell. 1989. Differential transport requirements of HLA and H-2 class I glycoproteins. Immunogenetics. 29:380–388.

27. Gould, K.G., H. Scotney, and G.G. Brownlee. 1991. Characterization of two distinct major histocompatibility complex class I Kk-restricted T-cell epitopes within the influenza A/PR/8/34 virus hemagglutinin. J. Virol. 65:5401–5409.

28. Bastin, J., J. Rothbard, J. Davey, I. Jones, and A. Townsend. 1987. Use of synthetic peptides of influenza nucleoprotein to define epitopes recognized by class I-restricted cytotoxic T lymphocytes. J. Exp. Med. 165:1508–1512.

29. Hammerling, G., U. Hammerling, and H. Lemke. 1979. Isolation of twelve monoclonal antibodies against Ia and H-2 antigens. Serological characterisation and reactivity with B and T lymphocytes. Immunogenetics. 8:433–439.

30. Ortiz Navarrete, V., and G.J. Hammerling. 1991. Surface appearance and instability of empty H-2 class I molecules under physiological conditions. Proc. Natl. Acad. Sci. USA. 88:3594–3597.

31. Elliott, T., V. Cerundolo, J. Elvin, and A. Townsend. 1991. Peptide-induced conformational change of the class I heavy chain. Nature (Lond.). 351:402–406.

32. Cerundolo, V., T. Elliott, J. Elvin, J. Bastin, H.G. Rammensee,

and A. Townsend. 1991. The binding affinity and dissociation rates of peptides for class I major histocompatibility complex molecules. Eur. J. Immunol. 21:2069–2075.

33. Chakrabarti, S., K. Brechling, and B. Moss. 1985. Vaccinia virus expression vector: coexpression of beta-galactosidase provides visual screening of recombinant virus plaques. Mol. Cell. Biol. 5:3403–3409.

34. Elvin, J. 1992. Presentation of Peptide Antigens to Cytotoxic T Lymphocytes. Oxford University Press, Oxford, UK. p. 1.

35. Gould, K., J. Cossins, J. Bastin, G.G. Brownlee, and A. Townsend. 1989. A 15 amino acid fragment of influenza nucleoprotein synthesized in the cytoplasm is presented to class I-restricted cytotoxic T lymphocytes. J. Exp. Med. 170:1051–1056.

36. Anderson, K., P. Cresswell, M. Gammon, J. Hermes, A. Williamson, and H. Zweerink. 1991. Endogenously synthesized peptide with an endoplasmic reticulum signal sequence sensitizes antigen processing mutant cells to class I-restricted cell-mediated lysis. J. Exp. Med. 174:489–492.

37. Stoller, T.J., and D. Shields. 1989. The propeptide of preprosomatostatin mediates intracellular transport and secretion of alpha-globin from mammalian cells. J. Cell Biol. 108:1647–1655.

38. Lippincott Schwartz, J., L. Yuan, C. Tipper, M. Amherdt, L. Orci, and R.D. Klausner. 1991. Brefeldin A's effects on endosomes, lysosomes, and the TGN suggest a general mechanism for regulating organelle structure and membrane traffic. Cell. 67:601–616.

39. Yewdell, J.W., and J.R. Bennink. 1989. Brefeldin A specifically inhibits presentation of protein antigens to cytotoxic T lymphocytes. Science (Wash. DC). 244:1072–1075.

40. Nuchtern, J.G., J.S. Bonifacino, W.E. Biddison, and R.D. Klausner. 1989. Brefeldin A implicates egress from endoplasmic reticulum in class I restricted antigen presentation. Nature (Lond.). 339:223–226.

41. Weiss, S., and B. Bogen. 1991. MHC class II-restricted presentation of intracellular antigen. Cell. 64:767–776.

42. Michalek, M.T., B. Benacerraf, and K.L. Rock. 1992. The class II MHC-restricted presentation of endogenously synthesized ovalbumin displays clonal variation, requires endosomal/lysosomal processing, and is up-regulated by heat shock. J. Immunol. 148:1016–1024.

43. Gould, K.G., H. Scotney, A.R.M. Townsend, J. Bastin, and G.G. Brownlee. 1987. Mouse H-2k-restricted cytotoxic T cells recognize antigenic determinants in both the HA1 and HA2 subunits of the influenza A/PR/8/34 hemagglutinin. J. Exp. Med. 166:693–701.

44. Ortmann, B., M.J. Androlewicz, and P. Cresswell. 1994. MHC class I/beta-2 microglobulin complexes associate with TAP transporters before peptide binding. Nature (Lond.). 368:864–867.

45. Suh, W.K., M.F. Cohen-Doyle, K. Fruh, K. Wang, P.A. Peterson, and D.B. Williams. 1994. Interaction of MHC class I molecules with the transporter associated with antigen processing. Science (Wash. DC). 264:1322–1326. (Abstr.)

46. Blobel, G., and B. Dobberstein. 1975. Transfer of proteins across membranes. J. Cell Biol. 67:835–851.

47. van Binnendijk, R.S., C.A. van Baalen, M.C. Poelen, P. de Vries, J. Boes, V. Cerundolo, A.D.M.E. Osterhaus, and F.G.C.M. UytdeHaag. 1992. Measles virus transmembrane fusion protein synthesized de novo or presented in immunostimulating complexes is endogenously processed for HLA class I- and class II-restricted cytotoxic T cell recognition.

J. Exp. Med. 176:119–128.

48. Hammond, S.A., R.C. Bollinger, T.W. Tobery, and R.F. Silli- ciano. 1993. Transporter-independent processing of HIV-1 enve- lope protein for recognition by CD8+ T cells. *Nature (Lond.).* 364:158–161.

49. Wei, M.L., and P. Cresswell. 1992. HLA-A2 molecules in an antigen-processing mutant cell contain signal sequence-derived peptides. *Nature (Lond.).* 356:443–446.

50. Bonifacino, J.S., C.K. Suzuki, J. Lippincott Schwartz, A.M. Weissman, and R.D. Klausner. 1989. Pre-Golgi degradation of newly synthesized T-cell antigen receptor chains: intrinsic sensitivity and the role of subunit assembly. *J. Cell Biol.* 109:73–83.

51. Wileman, T., G.R. Carson, M. Concino, A. Ahmed, and C. Terhorst. 1990. The gamma and epsilon subunits of the CD3 complex inhibit pre-Golgi degradation of newly synthesized

T cell antigen receptors. *J. Cell Biol.* 110:973–986.

52. Wileman, T., L. Kane, and C. Tehorst. 1993. Degradation of T cell receptor chains in the endoplasmic reticulum is inhibited by inhibitors of cysteine proteases. *Cell Reg.* 2:753–765.

53. Tsao, Y., N. Ivessa, M. Adesnik, D. Sabatini, and G. Keibich. 1992. COOH terminally truncated forms of Ribophorin I are degraded in pre-Golgi compartments by a calcium-dependent process. *J. Cell Biol.* 116:57–67.

54. Wikstrom, L., and H.F. Lodish. 1991. Nonlysosomal, pre-Golgi degradation of unassembled asialoglycoprotein receptor subunits: a TLCK- and TPCK-sensitive cleavage within the ER. *J. Cell Biol.* 113:997–1007.

55. Wassler, M., and E. Fries. 1993. Proteolytic cleavage of hap- toglobulin occurs in a subcompartment of the endoplasmic retic- ulum: evidence from membrane fusion in vitro. *J. Cell Biol.* 123:285–291.