Class I Molecules Retained in the Endoplasmic Reticulum Bind Antigenic Peptides

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Summary

We isolated major histocompatibility complex (MHC)-specific viral peptides from cells infected with influenza virus in the continuous presence of the drug brefeldin A, which blocks exocytosis of newly synthesized MHC class I molecules. MHC-specific peptides were also isolated from cells expressing mouse K^d class I MHC molecules whose cytoplasmic domain was substituted by that of the adenovirus E3/19K glycoprotein. This molecule was retained in an intracellular pre–Golgi complex compartment as demonstrated by immunocytochemical and biochemical means. Since we show that intracellular association of antigenic peptides with such retained class I molecules is necessary for their isolation from cellular extracts, this provides direct evidence that naturally processed peptides associate with class I MHC molecules in an early intracellular exocytic compartment.

$CD8^+$ T lymphocytes (T_{CD8+}) play an important role in eradicating intracellular parasites (viruses and unicellular organisms) and neoplasms (1). T_{CD8+} recognize class I molecules of the MHC bearing peptides of 8–10 residues derived from antigenic proteins (2, 3). Class I molecules consist of a 43-kD polymorphic transmembrane α chain noncovalently associated with 12-kD β_2-microglobulin. Chain assembly occurs within minutes after biosynthesis, probably within the endoplasmic reticulum (ER).[1] Class I molecules are then transported to the cell surface via the Golgi complex and post–Golgi complex vesicles.

A critical issue in understanding class I–mediated antigen presentation is the identity of the compartment in which class I molecules associate with antigenic peptides. Through the use of mutant cells it has been established that at least two gene products (termed TAP1 and TAP2 [transporter associated with antigen processing]) encoded by the MHC are needed for efficient presentation of biosynthesized antigens. These proteins are thought to form a transporter capable of conveying antigenic peptides from the cytosol to an intracellular exocytic compartment (4–7). In TAP-deficient cells, class I molecules are often retained in the ER (8–11). This conclusion is consistent with findings that presentation of biosynthesized antigens is blocked by agents that interfere with the egress of class I molecules from early exocytic compartments.

One such agent is the fungal metabolite brefeldin A (BFA). Upon addition of BFA to cells, the Golgi complex rapidly disassembles and is transported, at least in part, in a retrograde manner to the ER (12–16). Newly synthesized membrane or secretory proteins are retained in an early secretory compartment in BFA-treated cells. BFA completely blocks the ability of virus-infected cells to present antigens to virus-specific T_{CD8+} (17, 18). Another agent that blocks antigen presentation is the adenovirus E3/19K membrane glycoprotein, which binds class I molecules (without blocking peptide association) and retains them in the ER by virtue of a sequence in its COOH-terminal cytosolic tail (19–27). The inhibitory effects of BFA and E3/19K on antigen presentation have two general explanations: either these agents prevent class I molecules from reaching a distal secretory compartment where antigen association occurs, or they prevent the egress of class I molecules that are bound to antigenic peptides.

To distinguish between these possibilities, we determined whether naturally processed viral peptides could associate with class I molecules retained intracellularly through the action of BFA or due to the replacement of the cytosolic domain with that of E3/19K. Peptide association was measured using the method described by Rötzschke et al. (28, 29), in which acid-soluble peptides of <5 kD are separated via reverse-phase HPLC. We demonstrate that the recovery of peptides by this method requires that the peptides bind to the appropriate class I molecule in an intracellular compartment, and is, therefore, a valid method of examining whether peptides can associate with intracellular forms of class I molecules.

[1] *Abbreviations used in this paper:* BFA, brefeldin A; ER, endoplasmic reticulum; HA, hemagglutinin; NP, nucleoprotein; VV, vaccinia virus.

Materials and Methods

Cells and Virus. RMA cells (H-2^b) (generously provided by Dr. K. Karre, Karolinska Institute, Stockholm, Sweden) were maintained in RPMI 1640 supplemented with 7.5% (vol/vol) FCS in an air/CO_2 (94/6%) atmosphere. L929 cells (H-2^k) were maintained in DMEM supplemented with 7.5% (vol/vol) FCS in an air/CO_2 (91/9%) atmosphere. The influenza virus A/PR/8/34 (PR8) was propagated in the allantoic cavities of 10-d-old chicken eggs and stored as infectious allantoic fluids. Recombinant vaccinia viruses (VV) were propagated as described (30, 31). The production and characterization of VV expressing influenza virus nucleoprotein (NP), hemagglutinin (HA), and H-2 K^d have been described (32, 33). The production and characterization of VV expressing an ER-retained form of K^d will be described in detail elsewhere (Bačík et al., manuscript in preparation).

Mice and Generation of CTL. 6–8-wk-old C57BL/6 (H-2^b) or BALB/c (H-2^d) mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Mice were immunized by intravenous injection with 5×10^8 PFU of VV recombinants. T_{CD8+} were generated from splenocytes derived from animals immunized with VV recombinants 2–6 wk previously by 7-d in vitro stimulation with PR8-infected autologous splenocytes as described (30, 31). In this manner HA-specific T_{CD8+} were generated from mice immunized with VV-HA, and NP-specific T_{CD8+} were generated from mice immunized with VV-NP. Clone 17 is an NP-specific, D^b-restricted clone produced as described (34).

Peptide Isolation and Cytotoxicity Assays. To isolate PR8 peptides, 10^9 RMA cells were infected for 8 h at 37°C with 25,000 HAU of PR8. To isolate peptides from VV-infected cells, 5×10^8 cells were infected for 5 h at 37°C at a multiplicity of infection of 5 PFU for each VV recombinant. BFA was added to cells at 5 μg/ml 10 min after the addition of virus and was maintained throughout the infection. Peptides were isolated essentially as described (35). Briefly, cells were Dounce homogenized in 20 ml 0.1% TFA at room temperature, sonicated in the cold, rotated for 30 min at 4°C, and centrifuged at 10,000 rpm in an SW28 rotor (Beckman Instrs. Inc., Palo Alto, CA) for 15 min. Supernatants were dried overnight in a Speedvac (Savant Instrs., Inc., Farmingdale, NY), and pellets dissolved in 0.1% TFA were chromatographed using a G-25 column (bed volume, 100 ml; eluent, 0.1% TFA/water; flow rate, 1.0 ml/min). Material of <5 kD was dried, suspended in 200 μl 0.1% TFA, and subjected to reverse-phase HPLC. Samples were chromatographed using a Delta Pak C-18, 300-Å column (3.9 mm × 30 cm with 15-μm spherical particles; Waters Associates, Milford, MA). The following gradient was used: eluent 1, 0.1% TFA/water; eluent 2, 0.1% TFA/acetonitrile (1 ml/min, 1-ml fractions); gradient: 0 min, 95% 1 and 5% 2; 30 min, 50% 1 and 50% 2; 45 min, 0% 1 and 100% 2; 50–60 min, 95% 1 and 5% 2. Fractions were dried overnight and suspended in 200 μl PBS. To test for the presence of antigenic peptides, 10 μl was added to 10^4 ^{51}Cr-labeled cells in 100 μl Iscove's modified DMEM supplemented with 7.5% FCS (IDMEM), and T_{CD8+} were added in 100 μl IDMEM. After a 4-h incubation at 37°C, 100 μl of supernatant was harvested and the amount of released ^{51}Cr determined by gamma counting. Percent specific release was calculated as: 100× [(experimental cpm − spontaneous cpm)/(total cpm − spontaneous cpm)].

Biochemical Methods. 2×10^7 L929 cells infected for 1 h with VV were incubated for 30 min in 2 ml methionine-free DMEM for 30 min at 37°C. After adding 200 μCi [^{35}S]methionine, cells were incubated for an additional 5 min at 37°C. After washing, cells were incubated for 0–3 h at 37°C before incubation at 0°C. Cells were then incubated for 20 min at 0°C with 0.14 M NaCl, 1 mM EDTA, 1% Triton X-100, 1 mM PMSF, 10 mM Tris HCl, pH 7.4, and the nuclei pelletted by centrifugation at 15,000 *g*. Supernatants were incubated with protein A–Sepharose preloaded with the anti-K^d mAb, SF 1.1.1 (HB-159; American Type Culture Collection, Rockville, MD). After extensive washing, class I molecules were eluted from beads by boiling in 0.1 M sodium acetate, 3 mM EDTA, 0.25% SDS, pH 6. Samples were split in half, incubated overnight at 37°C with or without 2 mU endo-β-*N*-acetylglucoseaminidase H (Endo H), and analyzed by SDS-PAGE. After fixing and staining with Coomassie blue, gels were incubated with Amplify (Amersham Corp., Arlington Heights, IL), dried under vacuum, and exposed to preflashed film. Fluorographs were developed using an automated film processor.

Immunocytochemical Methods. VV-infected cells were fixed by incubating with 1% (wt/vol) paraformaldehyde in PBS for 20 min and permeabilized by including saponin (0.1% [wt/vol]) in all subsequent steps. Cells were incubated for 30 min at 0°C with PBS containing 2% BSA and 20% hybridoma tissue culture supernatant, washed, and incubated with FITC-conjugated rabbit anti-mouse IgG for an additional 30 min. Washed cells were analyzed using an Epics Profile flow cytometer. To localize intracellular class I molecules, L929 cells grown on acid-cleaned coverslips infected for 5 h with VV were fixed with 3% paraformaldehyde and permeabilized as above. Cells were incubated first with a rabbit antisera specific for a protein localized to the medial-Golgi complex (36), and after washing, with Texas red–conjugated donkey anti-rabbit IgG and FITC-conjugated SF 1.1.1 (Pharmingen, San Diego, CA). Coverslips were mounted with Mowiol containing 5% (wt/vol) diazobicyclo-octane, and observed using a laser scanning confocal microscope (LCSM; MRC600; Bio-Rad Laboratories, Richmond, CA). Red and green fluorescent images were simultaneously acquired using the fast photon counting mode. Controls established that red and green fluorescence did not bleed into the inappropriate channel. Images were further manipulated using a Macintosh computer and Adobe Photoshop (Adobe Systems Incorp., Mountain View, CA) to alter eight-bit black-and-white images into eight-bit red and green images that were then merged to demonstrate the relationship between staining patterns. Prints were produced by a Kodak XL7700 color printer.

Results

Recovery of Antigenic Peptides from BFA-treated Cells. RMA cells (a lymphoid cell line derived from H-2^b mice) were infected for 8 h with PR8, and acid-soluble peptides derived from the cells were fractionated by HPLC and tested for their ability to sensitize ^{51}Cr-labeled RMA cells for lysis by polyclonal D^b-restricted, influenza virus NP-specific T_{CD8+}, or clone 17, a D^b-restricted, NP-specific T_{CD8+} clone (Fig. 1). Lysis was not observed in the absence of T_{CD8+}, which indicates that the fractions do not contain material capable of inducing ^{51}Cr release. Fractions 14 and 15 were found to sensitize cells for lysis by NP-specific T_{CD8+}. Occasionally, peptide was recovered from fraction 10, but in these instances >95% of the activity was present in fractions 14 and 15. This correlates with the recovery of virtually all of the antigenic activity in fractions 14 and 15 after identical HPLC analysis of a synthetic peptide corresponding to NP 366–374 added to extracts of uninfected RMA cells (not shown). This peptide is known to represent the naturally processed peptide recognized by D^b-restricted, NP-specific T_{CD8+} (28, 35).

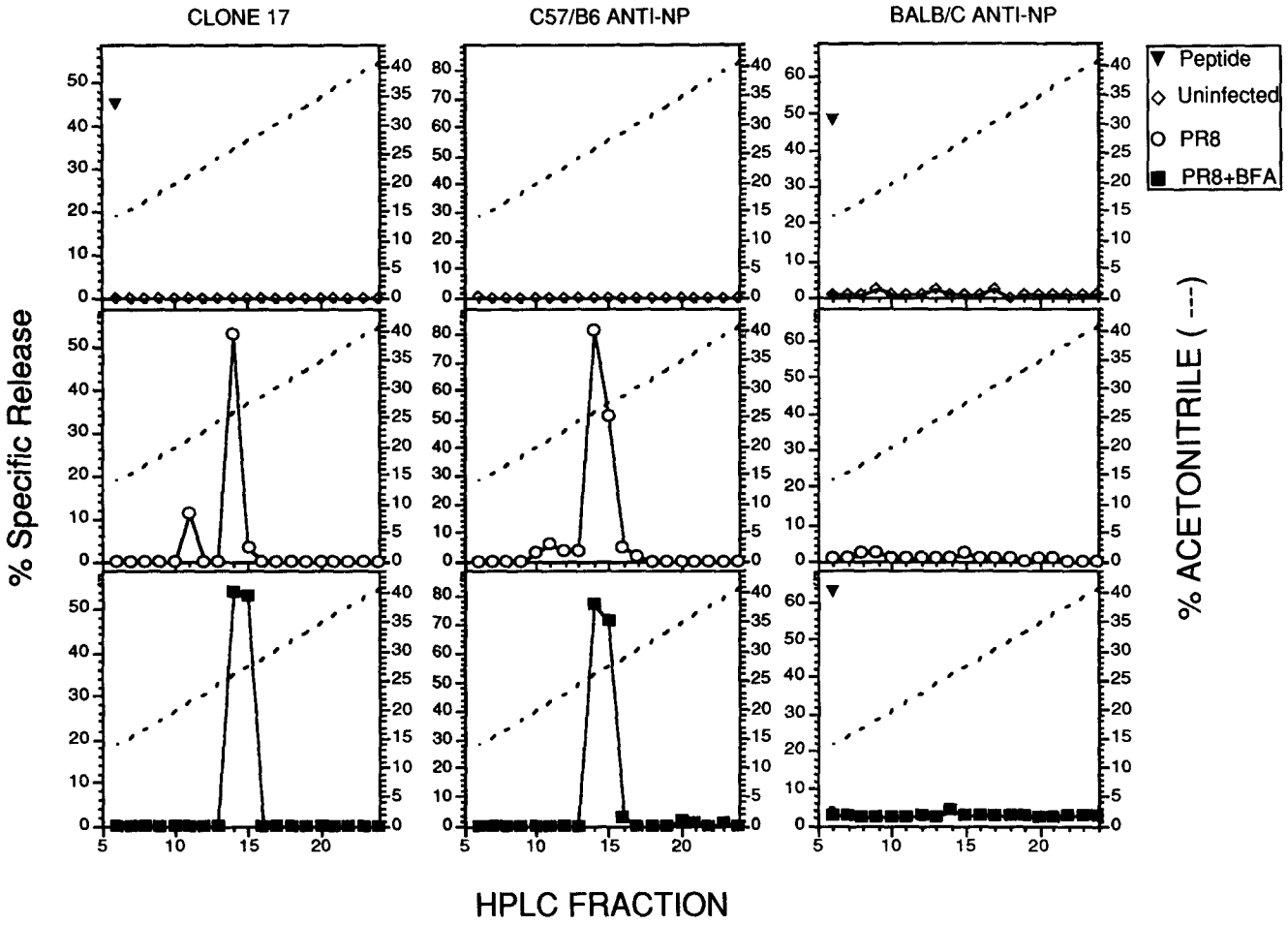

Figure 1. Effect of BFA on the recovery of influenza virus NP peptides from RMA cells. HPLC fractions from uninfected RMA cells (◇), or RMA cells infected with PR8 in the absence (○) or presence (■) of BFA, were tested for their ability to sensitize RMA cells (*left* and *middle*), or P815 cells (*right*) to lysis by various T_{CD8+}. Effector cells are listed above the respective panel. For positive controls, RMA cells were incubated with the NP 366–374 synthetic peptide, and P815 cells were incubated with the NP 147–155 synthetic peptide (▼). For negative controls, cells were incubated with saline; this resulted in <4% lysis.

Fractions derived from uninfected cells failed to sensitize RMA cells for lysis by NP-specific T_{CD8+}, demonstrating the requirement for viral gene expression in peptide formation. Fractions from PR8-infected cells did not sensitize P815 cells ($H\text{-}2^d$) for lysis by $H\text{-}2^d$-restricted, virus-specific T_{CD8+}, providing one measure of the MHC dependence of peptide existence in RMA cells (not shown). Another measure of the MHC dependence of peptide isolation is the failure of NP-specific T_{CD8+} from BALB/c mice ($H\text{-}2^d$) to recognize cells sensitized with HPLC fractions from PR8-infected RMA cells, despite their ability to lyse cells sensitized with a synthetic peptide consisting of residues 147–155, known to represent the naturally processed determinant from NP recognized in association with K^d (Fig. 1).

In the same experiment, cells were infected for 8 h in the continuous presence of BFA. To ensure that BFA was active in blocking antigen presentation, small aliquots of cells were labeled with ^{51}Cr and tested for recognition by clone 17 cells. This confirmed that BFA-treated cells failed to display sufficient class I–peptide complexes on their surfaces to enable T_{CD8+} recognition (not shown). Despite the failure of BFA-treated cells to present NP to T_{CD8+}, it was possible to recover a considerable amount of NP peptide from the cells. Peptides were present only in fractions 14 and 15 (Fig. 1). As with non–BFA-treated cells, antigenic activity recovered from cells was MHC restricted, since none of fractions sensitized P815 cells for recognition by $H\text{-}2^d$-restricted T_{CD8+} (not shown).

To compare the amounts of peptides recovered from BFA-treated and untreated RMA cells, twofold dilutions of peptide-containing fractions were tested for their ability to sensitize cells for lysis by clone 17 cells. In two completely independent experiments, the amount of peptide recovered from BFA-treated cells was 20 and 50% of that obtained from untreated cells (not shown). In a parallel experiment we determined that BFA has some inhibitory effect on viral gene expression in RMA cells. After 8.5 h of infection, BFA-treated cells expressed 25% the amount of NP as untreated cells, as deter-

mined by an ELISA of cell extracts using a NP-specific mAb (not shown). Thus, taking into account the decreased pool of NP, we conclude first, that BFA does not effect the generation of antigenic peptides or their association with class I molecules in influenza virus–infected RMA cells, and second, that intracellular peptide–class I complexes formed under these conditions are relatively stable.

To generalize our findings, we performed a similar experiment using P815 (a mastocytoma derived from H-2^d mice) infected with PR8 in the presence or absence of BFA (Fig. 2). Cell-derived acid-soluble peptides fractionated by HPLC were tested for their ability to sensitize P815 cells for lysis by H-2 K^d-restricted T_{CD8+} specific for HA or NP. As above, NP-specific effectors recognized peptides present in a single peak. By contrast, HA-specific effectors recognized peptides in two distinct peaks, which presumably contain two distinct peptides recognized by the polyclonal HA-specific T_{CD8+} populations. Infection of cells in the presence of BFA had little effect on the isolation of peptides, as antigenic peptides were recovered in the same fractions at roughly the same amounts. As above, the effectiveness of BFA at blocking antigen presentation was confirmed by testing an aliquot of cells used to isolate peptides in a standard ^{51}Cr release assay using influenza virus–specific T_{CD8+} (not shown). The MHC-dependent nature of peptide recovery was confirmed by the failure of the extracts to sensitize RMA cells for lysis by D^b-restricted, NP-specific T_{CD8+} (not shown).

Recovery of Antigenic Peptides from Class I Molecules with an ER Retention Sequence. We further studied peptide association using cells infected with a VV expressing a modified form of K^d, whose cytosolic domain was replaced with that of E3/19K (termed EC15 K^d). Transfer of the cytosolic domain of E3/19K to the COOH terminus of other membrane proteins has been shown to result in their retention in the ER (37). We initially compared the intracellular transport of EC15 K^d to wild-type K^d by immunoprecipitation of K^d from detergent extracts of cells radiolabeled with [^{35}S]methionine for 5 min and chased for up to 3 h. Immunoprecipitated molecules were treated with Endo H to determine whether their N-linked oligosaccharides were exposed to the modifying enzymes of the medial-Golgi complex, whose actions result in resistance of oligosaccharides to Endo H digestion. This revealed that while most wild-type K^d molecules became resistant to Endo H digestion 3 h after synthesis, all EC15 K^d detected throughout the chase period remained sensitive to Endo H digestion (Fig. 3). We further localized EC15 K^d in fixed and permeabilized cells by double immunofluorescence. While K^d clearly could be observed on the cell surface (not shown) or in the Golgi complex (Fig.

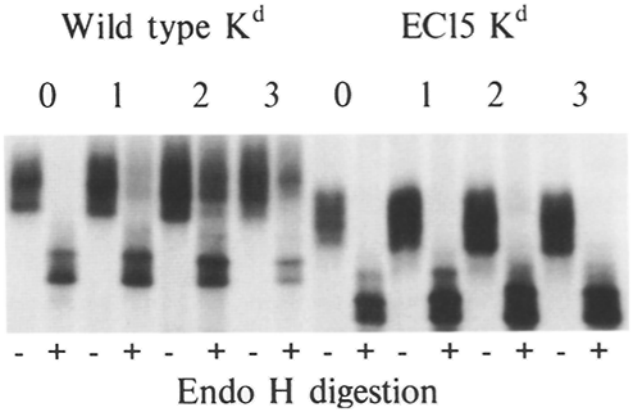

Figure 3. Intracellular transport of class I molecules. L929 cells infected with K^d-VV or EC15 K^d-VV were radiolabeled for 5 min with [^{35}S]methionine and chased for 0, 1, 2, and 3 h at 37°C. Detergent extracts were immunoprecipitated with a K^d-specific mAb. Half of the immunoprecipitates were digested with Endo H (+). Displayed is a fluorograph of immunoprecipitates analyzed by SDS-PAGE. We are uncertain as to why both wild-type K^d and EC15 K^d run as doublets. It cannot be attributed to differences in N-linked glycosylation, since molecules deglycosylated by Endo H display a similar pattern. It is also unlikely to represent trimming at the COOH terminus since this would be expected to allow EC15 K^d to leave the ER, or at the NH_2 terminus, since this would probably interfere with the folding necessary for interaction with the mAb used.

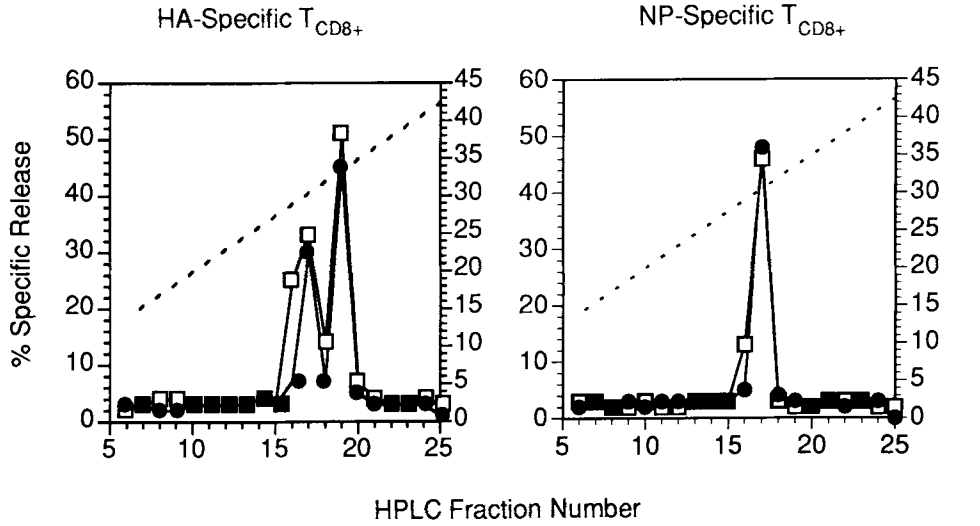

Figure 2. Effect of BFA on the recovery of influenza virus NP and HA peptides from P815 cells. Acid-soluble antigenic peptides from P815 cells infected with PR8 in the absence (□) or presence (●) of BFA were recovered and HPLC fractionated as described in Materials and Methods. BALB/c T_{CD8+} were derived from mice with a VV recombinant expressing HA or NP and stimulated in vitro with PR8-infected autologous cells. T_{CD8+} generated in this manner have been shown to be specific for the influenza virus gene product expressed by the VV recombinant used for priming (24).

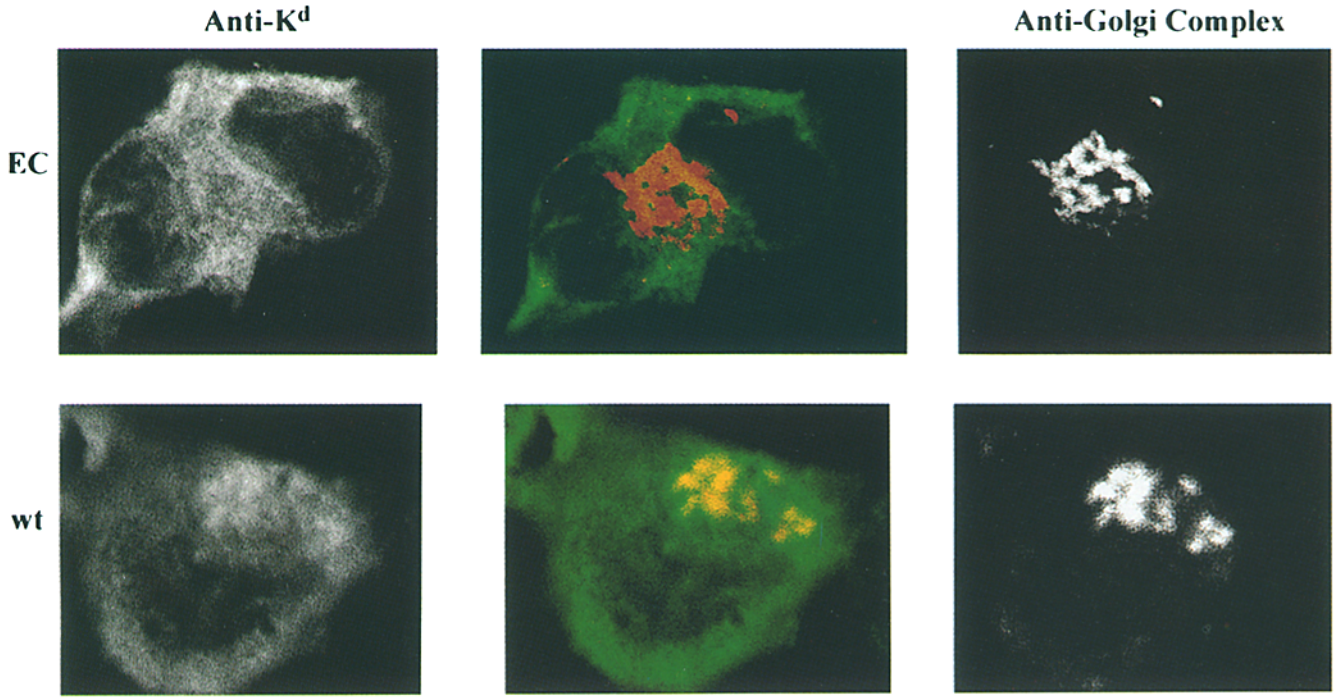

Figure 4. Immunofluorescent localization of K^d molecules. L929 cells infected with EC15 K^d VV (*top*) or wild-type K^d-VV (*bottom*) were fixed and permeabilized and stained for K^d (*left*) or the resident medial-Golgi complex protein MG160 (*right*). The relationship between the patterns is shown in the middle by coloring K^d fluorescence green and MG160 fluorescence red and merging the images. This demonstrates that the distribution of EC15 K^d, which is typical of the ER, is not related to the Golgi complex. By contrast, in addition to its ER distribution, wild-type Kd colocalizes with the Golgi complex, as clearly visualized in the intensely yellow areas of the merged image. Images were acquired with a scanning laser cofocal microscope.

4, *bottom*), in addition to the ER, EC15 was only detected in the ER (Fig. 4, *top*). The failure of EC15 K^d to reach the plasma membrane in appreciable amounts was confirmed by cytofluorography (Table 2, *nonpermeabilized*). These findings indicate that EC15 K^d is retained in an early secretory compartment, probably the ER.

Table 1. *Presentation of NP by L929 Cells Infected with Recombinant VVs*

	Percent specific ^{51}Cr release	
L929 Cells	40:1	13:1
Uninfected	2	1
NP-VV	0	0
K^d-VV	4	2
K^d-VV + NP-VV	69	53
EC15 K^d-VV	1	0
EC15 K^d-VV + NP-VV	4	3

Uninfected cells or cells infected for 5 h were tested in a 5-h ^{51}Cr release assay at the E/T ratios indicated. K^d-restricted T_{CD8+} specific for NP were generated as described (49).

The intracellular retention of EC15 K^d was further demonstrated functionally. L929 cells coinfected with EC15 K^d-VV and a VV recombinant expressing NP (NP-VV) were not recognized by polyclonal K^d-restricted, NP-specific T_{CD8+} as determined by a standard ^{51}Cr release assay, while cells coinfected with NP-VV and a VV recombinant expressing unmodified K^d (K^d-VV) were efficiently lysed by the same T_{CD8+} population (Table 2). Cytofluorographic analysis of permeabilized cells indicated that this cannot be attributed to lack of expression of NP or K^d (Table 1). Most importantly, equivalent amounts of peptide were recovered from cells expressing K^d and EC15 K^d (Fig. 5). Antigenic peptides were not recovered from cells infected with only EC15 K^d-VV, establishing that the peptides were derived from NP. The class I–specific nature of peptide isolation was demonstrated by our failure to isolate antigenic peptides from cells infected with only NP-VV. In an additional experiment, NP-VV-infected L929 cells were mixed with EC15 K^d VV-infected L929 cells, or with P815 cells infected with HA-VV, and the mixtures were processed for peptide isolation. As a positive control, peptides were also extracted from L929 cells coinfected with EC15 K^d-VV and NP-VV. Peptides were only recovered from cells simultaneously expressing NP and K^d (Fig. 6). Taken together, these findings extend prior findings by first, providing a direct demonstration that class I molecules are the only MHC gene product necessary for peptide isolation, and second, establishing that peptides re-

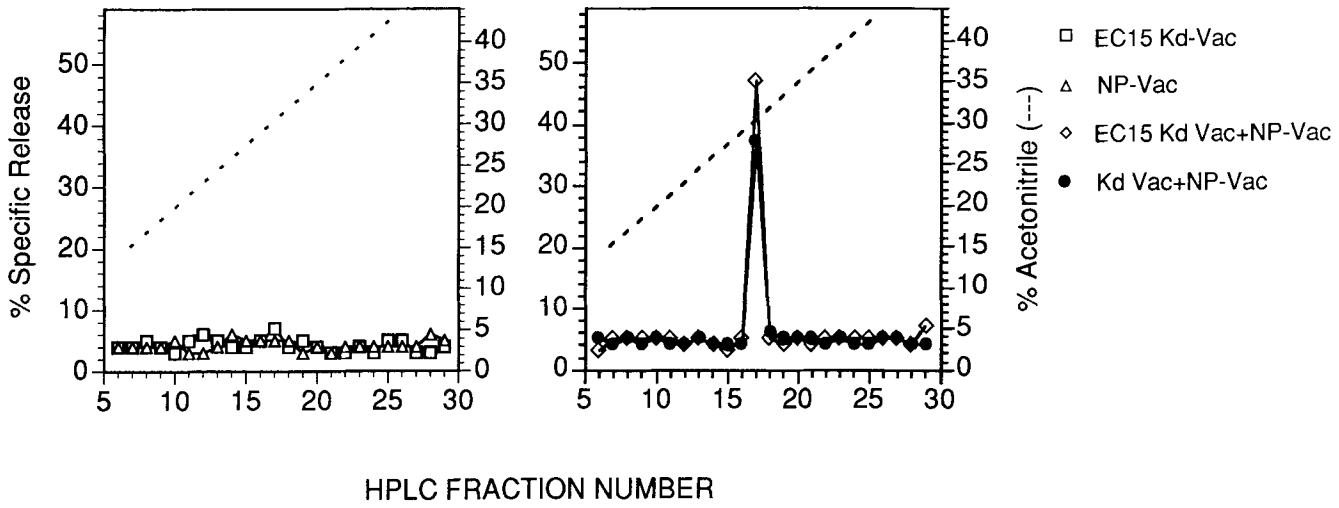

Figure 5. Isolation of K^d-associated NP peptides from L929 cells infected with VV expressing normal or ER-retained K^d molecules. Antigenic peptides were recovered from L929 cells infected with each of the following vaccinia viruses: NP-VV (△); EC15 K^d-VV(cytosolic tail replaced by the 15 COOH-terminal residues from the adenovirus E3/19K glycoprotein) (□); EC15 K^d-VV + NP-VV (◇); or K^d-VV + NP-VV (●). Peptides were used to sensitize P815 cells for lysis by NP-specific T_{CD8+} derived from BALB/c mice.

covered from cells are derived from peptide–class I complexes formed intracellularly, and not in lysis buffer during or after cell disruption.

Discussion

Del Val et al. (38) recently reported that cells infected with murine cytomegalovirus contained antigenic peptides derived from a viral protein despite the fact that the cells failed to display sufficient peptide–class I complexes on the cell surface to enable lysis by T_{CD8+} specific for the peptide. They also noted that cytomegalovirus infection hindered the transport of newly synthesized class I molecules, as determined by the sensitivity of N-linked oligosaccharides to digestion with Endo H. These findings indicate that under these conditions, antigen association occurs before the passage of class I molecules through the medial-Golgi complex, which is the presumed location of Golgi mannosidase II, whose action generally renders N-linked oligosaccharides resistant to digestion with Endo H.

In the present study we confirm by two different strategies the general finding that peptides can associate with class I molecules in an intracellular location, and extend this finding to include at least three more viral determinants (four if HA has two distinct determinants recognized in association with K^d). First, we demonstrated that antigenic peptides can be recovered from BFA-treated cells. Since BFA renders the cells insensitive to lysis by T_{CD8+} specific for the peptides, we conclude that the peptides are derived from intracellular class I molecules. This finding directly demonstrates that the myriad effects of BFA on intracellular membranes do not grossly affect either the formation of antigenic peptides, or their transport

Table 2. *Cytofluorographic Analysis of VV-infected L929 Cells Used for Peptide Isolation*

	Permeabilized				Nonpermeabilized	
	Anti-K^d		Anti-NP		Anti-K^d	
	Percent positive	MCF	Percent positive	MCF	Percent positive	MCF
NP-VV	3.5	21.5	94.2	60.6	3.5	6.6
EC15K^d-VV	85.7	43.0	3.92	16.5	2.4	6.2
EC15K^d-VV + NP-VV	69.1	29.6	90.1	55.3	3.7	6.4
K^d-VV + NP-VV	82.5	30.1	93.5	62.0	53.6	6.7

L929 cells infected for 5 h with the indicated recombinant VV were indirectly stained with mAbs specific for K^d or NP and analyzed using a flow cytometer. To detect intracellular antigens, cells were aldehyde fixed and permeabilized using saponin. To detect antigens on the cell surface, viable cells were used. Data are expressed as percent positive relative to cells stained with only the second fluorescent antibody, and the mean channel fluorescence (MCF) of positive cells.

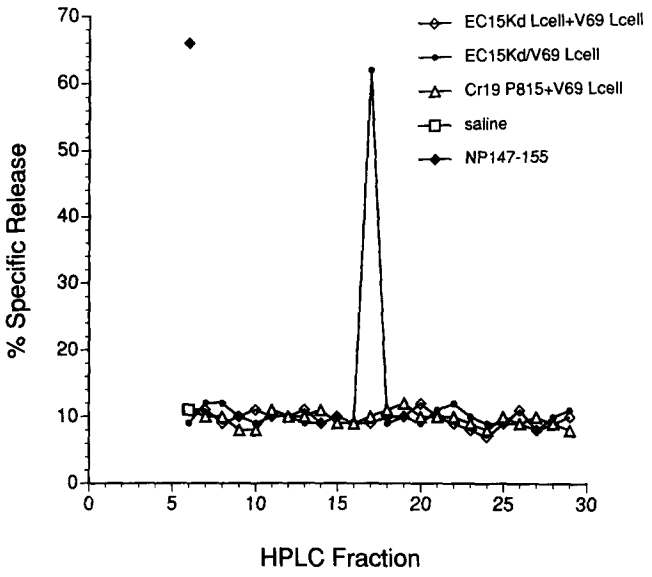

Figure 6. Isolation of NP peptides from cells expressing K^d requires the simultaneous expression of K^d and NP in the same cell. L929 cells were infected with NP-VV and were mixed with L929 cells infected with EC15 K^d-VV (◇) or P815 ($H2^d$) cells infected with Cr19VV (△). In addition, L929 cells were simultaneously infected with NP-VV and EC15 K^d-VV (●). Antigenic peptides were isolated, and peptides in HPLC fractions, saline (□), or NP 147–155 synthetic peptide (◆) were used to sensitize P815 cells for lysis by NP-specific T_{CD8+} derived from BALB/c mice.

from the cytosol to class I molecules. Due to the disruption of both the early and later proteins of the secretory pathway by BFA, it is not possible to infer the site of association of peptide with class I molecules, other than to conclude that association occurs before the delivery of class I molecules to the most distal regions of the *trans*-Golgi complex (known as the *trans*-Golgi network), which has been reported to be resistant to the effects of BFA on exocytosis (39, 40).

To more precisely determine the site of peptide association with class I molecules, we genetically engineered a K^d molecule containing the ER retention sequence of the E3/19K glycoprotein. This molecule does not acquire Endo H resistance, consistent with retention in an early secretory compartment. Immunofluorescence localization further suggests that the molecule is genuinely retained in the ER. Thus our finding that cells expressing EC15 K^d do not present NP peptides to T_{CD8+}, yet contain similar amounts of antigenic peptides as cells expressing wild-type K^d, indicates that class I molecules can associate with peptides in the ER itself, or in the *cis*-Golgi network, which might play a role in maintaining the ER localization of EC15 K^d (11).

The localization of peptide association to the early secretory pathway is consistent with the immunocytochemical localization of TAP1 to the ER and *cis*-most elements of the Golgi complex (41). Moreover, it indicates that peptides delivered to the ER by the addition of signal sequences (42, 43), or indeed signal sequences themselves (44, 45), likely associate with class I molecules in the ER, and not in a more distal portion of the secretory pathway.

Based on coelution in HPLC of HA and NP antigenic peptides derived from BFA-treated and untreated cells, and NP peptides derived from K^d- and EC15 K^d–expressing cells, it is possible that peptides isolated from retained class I molecules are identical to those displayed on the cell surface for T_{CD8+} recognition. This would suggest either that intracellular trimming of peptides associated with class I molecules is not a major source of the 8–10mers that bind to class I molecules with highest affinity, or that trimming occurs very rapidly. On the other hand, under the conditions used for HPLC, longer synthetic peptides have been found to coelute with naturally isolated peptides. We found, for example, that synthetic peptides consisting of residues 366–377, 367–376, or 367–377 coeluted with 366–374 under these conditions. We observed in several experiments that NP peptides derived from BFA-treated RMA cells were more heavily represented in fraction 15 than 14, while peptides from nontreated cells had the opposite distribution, suggesting that these peptides are not identical. It should be possible to determine whether longer peptides are present on intracellular class I molecules via chemical analysis, as recently described (46), and thus directly assess the possibility that peptides are trimmed after binding to class I molecules.

The association of antigenic peptides with class I molecules in the ER is, in some respects, a curious evolutionary choice. First, the ER is voluminous (occupying ~10% of the volume of some cells), which would not favor the binding of low amounts of peptides if they were simply delivered to the ER lumen by the transport process. Second, peptide binding molecular chaperones (such as BiP/GRP 71) are present in the ER at very high concentrations (47, 48), and might be expected to compete for peptide binding with class I molecules. The ability of class I molecules to efficiently bind antigenic peptides in the face of these difficulties has several interesting possible explanations. (*a*) Antigen association occurs in subdomains in which class I and TAP molecules are concentrated, perhaps by being physically linked to one another (such links would have to be tenuous since coprecipitation has not been reported, even in crosslinking studies [49]); (*b*) antigenic peptides are selected for their low affinity for molecular chaperones; and (*c*) peptide transfer to class I molecules is facilitated by an unidentified gene product.

Finally, consider the possibility that the choice of the ER for antigen association is a remnant of the evolutionary origins of antigen processing. The first antigens presented by primordial class I molecules might have been signal peptides, or peptides produced by the proteolytic processes believed to occur in the ER. Later, TAP genes might have evolved to broaden the types of peptides presented by class I molecules by allowing class I molecules access to peptides present in the cytosol. It will be of interest to determine if nonmammalian MHCs encode TAP homologues. If organisms can be identified that lack TAP genes, it might be possible to observe the primordial antigen processing system at work.

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