Assembly of MHC Class I Molecules with Biosynthesized Endoplasmic Reticulum-Targeted Peptides Is Inefficient in Insect Cells and Can Be Enhanced by Protease Inhibitors

Yuping Deng,* James Gibbs,* Igor Bačík,* Angel Porgador,† James Copeman,‡ Paul Lehner,‡ Bodo Ortmann,‡ Peter Cresswell,‡ Jack R. Bennink,[1]* and Jonathan W. Yewdell[1]*

To study the requirements for assembly of MHC class I molecules with antigenic peptides in the endoplasmic reticulum (ER), we studied Ag processing in insect cells. Insects lack a class I recognition system, and their cells therefore provide a "blank slate" for identifying the proteins that have evolved to facilitate assembly of class I molecules in vertebrate cells. H-2K^b heavy chain, mouse β_2-microglobulin, and an ER-targeted version of a peptide corresponding to Ova$_{257–264}$ were expressed in insect cells using recombinant vaccinia viruses. Cell surface expression of K^b-OVA$_{257–264}$ complexes was quantitated using a recently described complex-specific mAb (25-D1.16). Relative to TAP-deficient human cells, insect cells expressed comparable levels of native, peptide-receptive cell surface K^b molecules, but generated cell surface K^b-OVA$_{257–264}$ complexes at least 20-fold less efficiently from ER-targeted peptides. The inefficient assembly of K^b-OVA$_{257–264}$ complexes in the ER of insect cells cannot be attributed solely to a requirement for human tapasin, since first, human cells lacking tapasin expressed endogenously synthesized K^b-OVA$_{257–264}$ complexes at levels comparable to tapasin-expressing cells, and second, vaccinia virus-mediated expression of human tapasin in insect cells did not detectably enhance the expression of K^b-OVA$_{257–264}$ complexes. The assembly of K^b-OVA$_{257–264}$ complexes could be greatly enhanced in insect but not human cells by a nonproteasomal protease inhibitor. These findings indicate that insect cells lack one or more factors required for the efficient assembly of class I-peptide complexes in vertebrate cells and are consistent with the idea that the missing component acts to protect antigenic peptides or their immediate precursors from degradation. ***The Journal of Immunology,*** **1998, 161: 1677–1685.**

Major histocompatibility complex class I molecules function to display short peptides on the cell surface for inspection by CD8$^+$ T cells. Peptides are usually between 8 and 11 residues in length and are derived largely from a cytosolic pool of polypeptides (1–3). The evolutionary value of the class I processing system derives from its ability to inform CD8$^+$ T cells of the presence of intracellular parasites, including viruses, bacteria, and eukaryotic parasites (4).

Class I molecules consist of two noncovalently bound subunits. The molecule is anchored to the membrane by the α-chain glycoprotein, which forms the bulk of class I molecules, including the peptide-binding groove. The conformational stability of α-chains is greatly enhanced by their assembly with β_2-microglobulin (β_2m),[2] a small, nonglycosylated soluble protein. Both α-chains and β_2m are cotranslationally inserted into the endoplasmic reticulum (ER), the site of folding and assembly of secreted and cell surface proteins. Like other ER-targeted proteins, α-chain folding is facilitated by ER-resident molecular chaperones. Nascent α-chains transiently associate with the ER-chaperone, calnexin (5, 6), and probably other chaperones as well (7–9). The association of α-chains with β_2m occurs while α-chains are bound to calnexin or surrogate chaperones. The folding of nascent β_2m has not been carefully studied, but probably also involves molecular chaperones, since following synthesis, its binding to α-chains is delayed by ~10 min. The association of class I molecules with β_2m is associated with the transfer of the complex to calreticulin, a molecular chaperone closely related to calnexin. The calreticulin class I-β_2m complex next binds to a recently discovered MHC gene product, termed tapasin (or TAP-A), which is required for the complex to subsequently bind to TAP (10–12). TAP functions to deliver cytosolic peptides to the ER (13), and it is presumed that such peptides preferentially associate with class I molecules bound to the TAP that transported the peptide. Peptide binding induces the release of class I molecules from TAP, although not necessarily from calnexin (14, 15), and the completed molecules are rapidly transported through the Golgi complex to the cell surface.

Despite the impressive gains in knowledge over the past decade regarding class I biosynthesis, it is uncertain whether all of the critical components of the ER machinery required for efficient assembly of class I heterotrimers have been identified. The most rigorous strategy for addressing this question is to reconstitute class I assembly in cells or cell-free systems derived from invertebrates, since all vertebrates possess a class I Ag processing system. It is obviously advantageous to use cells that express housekeeping proteins as similar as possible to mammalian cells.

Insects are phylogenetically close to vertebrates, and their cells are able to properly target to the ER, glycosylate, fold, and assemble most mammalian glycoproteins. Insect cells are often called on

Laboratories of *Viral Diseases and †Immunology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892; and ‡Section of Immunobiology, Howard Hughes Medical Institute, Yale University School of Medicine, New Haven, CT 06510

Received for publication December 16, 1997. Accepted for publication April 14, 1998.

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.

[1] Address correspondence and reprint requests to Dr. Jonathan W. Yewdell or Dr. Jack R. Bennink, Room 213, Building 4, National Institutes of Health Bethesda, MD. E-mail address: jyewdell@nih.gov or jbennink@nih.gov

[2] Abbreviations used in this paper: β_2m, β_2-microglobulin; ER, endoplasmic reticulum; VV, vaccinia virus; NP, nucleoprotein; ES, adenovirus 2 gp19 signal sequence; DPBS, Dulbecco's modified PBS; endo H, endoglycosidase H.

 0022-1767/98/$02.00

when large quantities of secreted mammalian proteins are needed. Jackson, Peterson, and their colleagues have pioneered the use of *Drosophila* cell lines expressing class I molecules from transfected genes (16, 17). This system has yielded important insights into Ag processing (as well as milligram quantities of peptide receptive class I molecules suitable for crystallization), but suffers from problems inherent to working with cloned cell lines and the difficulty of transfecting cells, particularly when it is necessary to express multiple genes. These problems can be avoided using viral vectors to transiently express components of the class I machinery, since it is possible to infect with multiple viruses to "mix and match" gene products of interest. Baculoviruses have been successfully used in this manner to express class I molecules (18, 19) and TAP in insect cells (20), but this vector is limited by its inability to infect mammalian cells.

In the present report, we show that recombinant vaccinia viruses (rVV) can be used to express class I molecules in insect cells. VV has the advantage of being the most commonly used viral vector for expressing foreign genes in mammalian cells, and has been the workhorse in identifying Ags recognized by $CD8^+$ T cells and in dissecting class I Ag processing mechanisms (21). Using rVVs expressing class I molecules and ER-targeted peptides, we demonstrate that mosquito cells efficiently synthesize peptide-receptive class I molecules in the ER and export them to the cell surface at a rate similar to mammalian cells. When it comes to assembling class I molecules with endogenously synthesized peptides, however, only ~3% or less of cell surface class I molecules contain peptides as opposed to ~40 to 80% of class I molecules expressed by human cells.

Materials and Methods

Cells

Aedes albopictus clone C6/36 cells (obtained from American Type Culture Collection (ATCC), Manassas, VA) were cultured at 27°C in L-15 Leibovitz medium supplemented with FBS (10%), nonessential amino acids, penicillin, and streptomycin. The .220 cells and .220 cells expressing HLA-B8 from a transfected gene (.220/B8) were generously provided by Dr. Thomas Spies (Fred Hutchinson Cancer Research Center, Seattle, WA). These cells and B3Z cells (22) were cultured in RPMI with 7.5% FBS at 37°C in a 94%:6% air:CO_2 atmosphere. T2 cells and L929 cells were cultured in DMEM with 10% FBS at 37°C in a 91%:9% air:CO_2 atmosphere.

Viruses

rVVs encoding the K^b α-chain, K^d α-chain, $ESOVA_{257–264}$, $ESNP_{147–155}$, and CD54 have been described (23, 24). A rVV expressing human tapasin under the control of the p7.5 early/late promoter was produced by inserting the tapasin cDNA into a modified form of pSC11. rVVs coexpressing β_2m with K^b, K^d, $ESOVA_{257–264}$, or $ESNP_{147–155}$ were produced as described by Coupar et al. (25). Briefly, the mouse β_2m^b gene under the control of the VV promoter P-F was inserted into plasmid TK-7.5A containing the herpes simplex virus type I thymidine kinase gene. This plasmid was inserted in rVVs by transfecting infected cells. Double recombinants were selected using aminopterin for thymidine kinase expression (26).

Infections

Aedes cells were dislodged from flasks by incubation with EDTA-containing PBS and vigorous tapping. After washing with Dulbecco's modified PBS (DPBS), cells were suspended at 10^7/ml PBS supplemented with 0.1% BSA (w/v), incubated with rVVs (10 plaque forming units/cell) for 1 h with gentle rocking at 27°C. Cells were diluted to 10^6 cells/ml in normal culture medium and incubated at 27°C for an additional 6 to 8 h with gentle rocking.

Flow cytometric analyses

Infected cells were washed with PBS and incubated with primary Abs for 20 min at room temperature. Ab binding was detected either using rabbit anti-mouse IgG conjugated to fluorescein (Dako, Carpinteria, CA) or in a three-step method using anti-mouse IgG1 conjugated to biotin (Jackson Immunoresearch, West Grove, PA) followed by streptavidin conjugated to phycoerythrin (Jackson Immunoresearch).

[^{35}S]Methionine labeling and immunoprecipitation

Aedes cells (10^7) were infected with rVVs as described above. Six hours postinfection, cells were washed twice with DPBS and once with methionine-free DMEM. Cells were resuspended and incubated in 4 ml methionine-free DMEM for 20 min at 27°C. The cells were resuspended in fresh methionine-free DMEM (250 μl), and 200 μCi of [^{35}S]methionine was added. Cells were incubated for 10 min at 27°C, washed with PBS, and then incubated with lysis buffer as described (23). mAb-reactive molecules were collected from postnuclear supernatants and analyzed by SDS-PAGE as described (23).

Quantitating β-galactosidase expression in B3Z cells

Aedes cells were infected with rVVs as described above. Six hours postinfection, cells were washed with PBS three times, and 1.25×10^5 cells were aliquoted into wells of 96-well plates. Equal numbers of B3Z cells were added and mixed well with the insect cells. Cells were cocultured for 8 to 12 h, washed with PBS, and incubated with phycoerythrin-conjugated anti-Thy1.2 Ab (PharMingen, San Diego, CA) to identify B3Z cells. After washing twice with DPBS, cells were suspended in 50 μl of PBS, and incubated at 37°C. Fifty microliters of chloromethylfluorescein di-β-D-galactopyranoside (Molecular Probes, Portland, OR) in water (1 μg/ml) was then added to each well. Five minutes later, 175 μl of PBS was added. Cells were pelleted, washed, incubated for 10 min at 37°C, and analyzed cytofluorographically. Live and phycoerythrin-positive cells were selected for analysis of green fluorescence.

Results

Expression of rVV encoded proteins by insect cells

We initially screened four insect cell lines available from the ATCC for their ability to express rVV-encoded early gene products. This was determined by immunofluorescence performed on live (for surface proteins) or fixed and permeabilized cells (for internal proteins). Live cells were analyzed by cytofluorography and fixed cells by microscopy. The highest levels of expression were obtained using *A. albopictus* clone c6/36a cells (termed *Aedes* cells throughout; additional ATCC-provided cell lines examined were CRL 1494, CRL 8003, and CRL 1711). Levels of expression varied considerably between individual cells, suggesting clonal variation in expression of VV-encoded genes. Subcloning of cells yielded a cell line that expressed rVV gene products more uniformly than the general population, and this clone was used throughout the studies described below.

Unlike mammalian cells, which are almost invariably killed by VV if they support expression of viral genes, the same *Aedes* cell line was previously shown by Franke and Hruby (27) to express early VV gene products and to remain viable. Late viral gene products are not expressed, and as cells continue to divide, viral proteins are eventually degraded. Monitoring the expression of recombinant cell surface proteins under the control of early and late promoters, we confirmed these findings. Expression of cell surface proteins under control of the p7.5 viral promoter was detected as early as 3 to 4 h postinfection, peaking at 6 to 8 h postinfection. We found that the levels of expression of rVV encoded genes are similar to that of mammalian cells when cell volume is factored in, and mammalian cells are infected at 27 to 28°C (higher temperatures induce a heat shock response in insect cells resulting in inhibition of VV gene expression).

Biosynthesis of K^b molecules in Aedes *cells*

To study the biosynthesis of H-$2K^b$ molecules in *Aedes* cells, cells were infected with rVV-expressing K^b α-chains and tested for cell surface expression of K^b by indirect immunofluorescence using the Y3 mAb. Y3 binding requires K^b to be in a native or near native conformation (28). At the temperature used for infection (28°C),

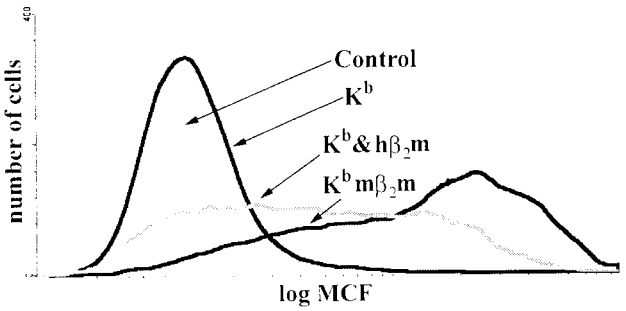

FIGURE 1. Cytofluorography of rVV-infected *Aedes* cells. The expression of K^b molecules on the surface of viable *Aedes* cells infected with the indicated rVV at 28°C was determined by indirect immunofluorescence using the Y3 mAb. MCF, mean channel fluorescence.

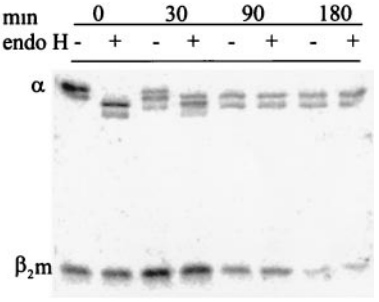

FIGURE 2. SDS-PAGE analysis of K^b molecules biosynthesized by *Aedes* cells. *Aedes* cells infected with VV-$K^b\beta_2$m were pulse radiolabeled with [^{35}S]methionine and chased for up to 180 min. Molecules reactive with Y3 were incubated without (−) or with (+) endo H and analyzed by SDS-PAGE.

K^b molecules do not require a high affinity ligand for stable expression on the surface of mammalian cells (29). As seen in Figure 1, expression of Y3-reactive K^b at the cell surface was negligible in the absence of endogenously synthesized β_2m. Coinfection with a rVV-expressing human β_2m resulted in expression of Y3-reactive K^b. This confirms numerous reports that expression of native K^b at the cell surface requires coexpression of β_2m (30–33). Coinfection with two rVVs to produce class I molecules resulted in expression in approximately half of the cells. To increase the efficiency of synthesizing $K^b\beta_2$m heterodimers, we constructed a rVV that coexpresses K^b with mouse β_2m. Infection with this rVV resulted in both higher levels of K^b expression and expression in a greater percentage of cells (Fig. 1).

We next biochemically examined the assembly and intracellular transport of K^b molecules. VV-$K^b\beta_2$m-infected cells were pulse radiolabeled for 10 min and chased for up to 3 h. All incubations were performed at 28°C. K^b molecules in detergent lysates were collected with immobilized Y3 and analyzed by SDS-PAGE. This resulted in the recovery of K^b α-chains and β_2m. Two distinctly migrating forms of K^b were recovered at all time points. We are uncertain whether this represents posttranslational modifications exclusively occurring in one of the species that increases or decreases its mobility, or, perhaps more likely in view of the rapidity of the process, the premature termination of the more rapidly migrating species.

The export of K^b molecules from the ER was monitored by endoglycosidase H (endo H) digestion K^b collected with Y3. In insect cells, *N*-linked oligosaccharides associated with glycoproteins are trimmed to endo H-resistant forms in the Golgi complex, but are not sialylated. Acquisition of endo H resistance is accompanied by increased mobility of glycoproteins in SDS-PAGE due to oligosaccharide trimming in the Golgi complex (sialylation is responsible for the decreased mobility of endo H-resistant K^b molecules observed in mammalian cells). In Figure 2, it can be seen that Y3-reactive-K^b molecules acquire endo H resistance with a $t_{1/2}$ of 30 min. This is approximately twice the rate observed in *Drosophila* cells (34) and similar to that observed in TAP-expressing mammalian cells (17, 35). This is especially notable because export from the ER of mammalian cells is slowed at 28°C. These findings demonstrate that the ER of *Aedes* cells is able to rapidly assemble and export class I molecules.

Also of interest in Figure 2 is the behavior of β_2m. Immediately following pulse labeling, radiolabeled β_2m is detected at ~60% of the maximal level collected (attained at the 30 min chase). This finding contrasts with those in mammalian cells in which detection of labeled β_2m in class I complexes occurs more slowly ($t_{1/2}$ of ~15 to 30 min postchase). This may reflect either a more rapid availability of newly synthesized β_2m in insect cells or the decreased size of the preexisting nonradiolabeled β_2m pool. It is also clear that radiolabeled β_2m dissociates from Y3-reactive K^b between 30 and 180 min following synthesis. β_2m also dissociates in mammalian cells and probably represents replacement of mouse β_2m at the cell surface with bovine β_2m present in the chase medium.

K^b molecules expressed by Aedes *cells are peptide receptive*

Cell surface K^b molecules lacking high affinity peptides denature at 37°C and no longer bind Y3 (28). As seen in Table I, incubation of VV-$K^b\beta_2$m infected *Aedes* cells for 60 min resulted in the loss of ~90% of Y3-reactive cell surface molecules. The few remaining molecules were resistant to a further 2-h incubation at 37°C. To determine whether cell surface K^b molecules synthesized at 28°C were capable of binding exogenous peptides, cells were exposed to a synthetic peptide (SIINFEKL) corresponding to residues 257–264 from OVA ($OVA_{257–264}$), known to bind K^b with high affinity (36–38). Peptide exposure occurred only at temperatures below 4°C, which prevents peptide from gaining access to intracellular K^b molecules (39). Approximately 65% of cell surface K^b molecules were stabilized by exposure to $OVA_{257–264}$. These molecules remained stable for 3 h at 37°C, demonstrating that most K^b molecules expressed at the surface of *Aedes* cells at 28°C are capable of binding antigenic peptides. Together with the biochemical findings presented above, these findings demonstrate that insect cells are capable of rapidly exporting newly synthesized class I molecules that are properly conformed and capable of binding peptides.

Loading of endogenous peptides in Aedes *cells: T cell activation*

We first examined the ability of rVV-infected *Aedes* cells to assemble endogenous K^b-$OVA_{257–264}$ complexes using B3Z T hybridoma cells. B3Z cells express β-galactosidase under control of the IL-2 promoter. Triggering of their TCR, specific for K^b-$OVA_{257–264}$ complexes, induces expression of β-galactosidase (22). B3Z cells were incubated with rVV-infected *Aedes* cells and their level of β-galactosidase was determined cytofluorographically following incubation with a substrate that becomes fluorescent after cleavage by β-galactosidase.

As seen in Figure 3, incubation of B3Z cells with *Aedes* cells infected with rVVs expressing a control glycoprotein (ICAM-1) were minimally stimulated whether the cells were incubated with a control peptide or with $OVA_{257–264}$. Incubation with *Aedes* cells infected with VV-$K^b\beta_2$m and pulsed with $OVA_{257–264}$ resulted in the induction of β-galactosidase. This finding establishes that *Aedes* cells are capable of stimulating T cell hybridomas, extending prior results demonstrating stimulation of T cells by

Table I. *Expression of thermostable cell surface K^b molecules by* Aedes *cells*[a]

Virus	Expt. Experiment	SIINFEKL	Time at 37°C 0	1 h	2 h	3 h
Uninfected	1	No	55	50	52	51
VV-$K^b\beta_2$m	1	No	508	97	82	75
VV-$K^b\beta_2$m	1	Yes	475	319	303	294
Uninfected	2	No	84			
VV-K^b and VV-β_2mESNP$_{147–155}$	2	No			104	
VV-K^b VV-β_2mESNP$_{147–155}$	2	Yes			424	
VV-K^b VV-β_2mESOVA$_{257–264}$	2	No			101	
VV-K^b VV-β_2mESOVA$_{257–264}$	2	Yes			411	

[a] *Aedes* cells were infected for 8 h at 28°C with the indicated rVV and then incubated at 4°C with or without SIINFEKL. Cells were washed and then incubated at 37°C for the indicated period. K^b expression was then determined by cytofluorography following indirect staining with Y3. Values represent mean channel fluorescence.

Drosophila cells expressing class I molecules from transfected genes (16, 41).

We next examined whether endogenously synthesized peptides could be loaded onto K^b molecules. To minimize the requirements for accessory Ag processing components, cells were coinfected with a rVV expressing $OVA_{257–264}$ delivered to the ER by the NH_2-terminal signal sequence of the adenovirus 2 E3/19K glycoprotein. This signal sequence has been shown to be active in insect cells (18). Mammalian cells efficiently load ER-targeted peptides (including $OVA_{257–264}$) in the absence of TAP (41) and are capable of producing tens of thousands of cell surface K^b-$OVA_{257–264}$ complexes (24). Cells coinfected with VV-$K^b\beta_2$m and VV-$ESOVA_{257–264}$ stimulated B3Z cells (Fig. 3*B*). The specificity of induction was shown by the similar lower levels of β-galactosidase induction when B3Z cells were incubated with *Aedes* cells coinfected with VV-$K^b\beta_2$m and VV-$ESNP_{147–155}$ (an ER-targeted K^d-binding peptide) or with VV-β_2m and VV-NP. Incubation of B3Z cells with a mixture of *Aedes* cells infected with either VV-$K^b\beta_2$m or VV-$ESOVA_{257–264}$ resulted in stimulation at a level similar to observed with VV-$K^b\beta_2$m alone (Fig. 3*B*, "Mix" bar). This indicates, first, that reinfection of B3Z cells by rVVs released from *Aedes* cells was not responsible for stimulation, and second, that association of K^b with $OVA_{257–264}$ occurred intracellularly in *Aedes* cells and not at the cell surface following secretion of $OVA_{257–264}$. As expected, stimulation of B3Z cells was not observed following coexpression of $K^b\beta_2$m with either a cytosolic version of the peptide (the E3/19K leader sequence replaced by Met), or full length OVA (not shown).

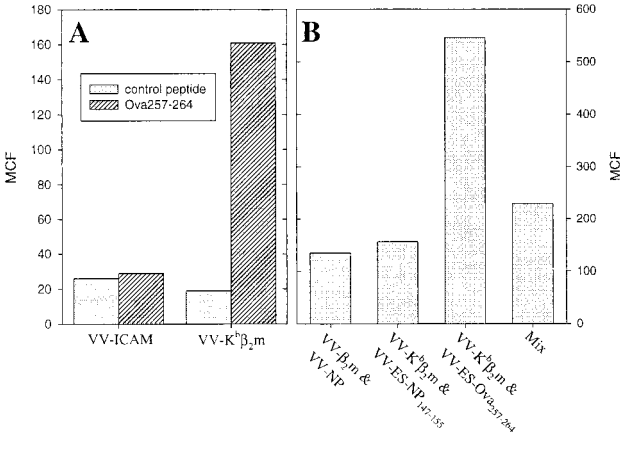

FIGURE 3. Activation of OVA-specific T hybridoma cells by rVV-infected *Aedes* cells. Activation of B3Z cells by rVV-infected *Aedes* cells was determined cytofluorographically following incubation of cells with a fluorogenic β-galactosidase substrate. Similar findings were made in two additional experiments.

Loading of K^b by endogenous ER-targeted $OVA_{257–264}$: detection by 25-D1.16 mAb

The findings obtained using the B3Z cells indicated that *Aedes* cells could produce K^b-$OVA_{257–264}$ complexes, but provided no idea as to the number of complexes expressed at the cell surface. That this number might be quite small was first indicated by Y3 mAb staining of cells coexpressing $K^b\beta_2$m with $ESOVA_{257–264}$. As seen in Table I, these cells failed to detectably express complexes stable at 37°C above control values obtained with cells coinfected with VV-$K^b\beta_2$m and a rVV expressing an ER-targeted K^d-binding peptide. In both cases, K^b molecules could be stabilized by the addition of exogenous $OVA_{257–264}$, demonstrating that native K^b molecules were produced in coinfected cells in this experiment.

To facilitate detection of low numbers of K^b-$OVA_{257–264}$ complexes, we used the 25-D1.16 mAb. This mAb is similar to a TCR in demonstrating high specificity for K^b-$OVA_{257–264}$ complexes (42), but has a higher affinity than typical TCRs, enabling it to detect complexes more sensitively than soluble TCRs. Indeed, its sensitivity for detecting class I peptide complexes approaches that of T cells (42). As seen in Figure 4 (*top panel*), binding of the 25-D1.16 mAb to VV-$K^b\beta_2$m-infected cells was easily detected following exposure of cells to $OVA_{257–264}$, providing direct confirmation that peptide induced stabilization of K^b to thermal denaturation reflects the presence of bound peptide.

Following coinfection of cells at 28°C with VV-$K^b\beta_2$m and VV-$ESOVA_{257–264}$, a small number of complexes could be detected at the cell surface by 25-D1.16 if a sensitive triple staining procedure was used (Fig. 4, *middle panel*). The same cells expressed considerable amounts of Y3-reactive K^b molecules (*bottom panel*). K^b-$OVA_{257–264}$ complexes were not detected following expression of either full length OVA or the cytosolic $OVA_{257–264}$ peptide (not shown).

These data indicate that *Aedes* cells are capable of producing small numbers of endogenous peptide class I complexes from endogenous peptides, but only if the peptides are delivered to the ER by a signal sequence.

Loading of K^b by endogenous ER-targeted $OVA_{257–264}$: comparison of Aedes *and mammalian cells*

The low numbers of K^b-$OVA_{257–264}$ complexes expressed on the *Aedes* cell surface contrasts greatly with their expression in mouse cells (>50,000 copies per cell) (42). It was plausible that the poor efficiency of peptide loading in insect cells was due strictly

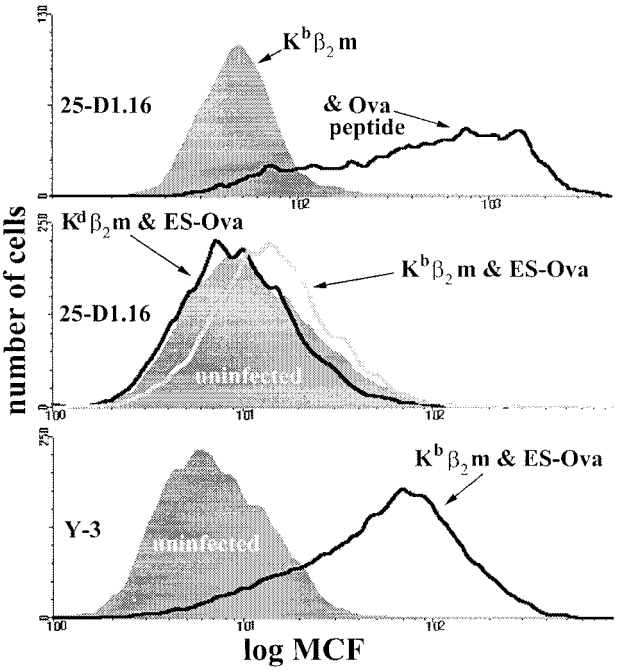

FIGURE 4. Cytofluorography of rVV-infected *Aedes* cells. The expression of K^b-$OVA_{257–264}$ complexes in *Aedes* cells infected with rVVs as indicated was determined cytofluorographically using the 25-D1.16 mAb in a triple sandwich method (*top two panels*). In the *top panel*, complexes were created by incubating cells with the $OVA_{257–264}$ synthetic peptide in the cold. In the *bottom panel*, K^b expression was determined using the Y3 mAb in a standard double sandwich assay. The lower sensitivity of this method accounts for the detection of lower number of molecules than with 25-D1.16 in the *middle panel*. In the *top panel*, control cells (gray histogram) are VV-$K^b\beta_2$m infected. In the *bottom two panels*, control cells are uninfected cells. In a large number of additional experiments, staining of uninfected cells was similar to staining of cells infected with control rVVs.

to the absence of TAP. To examine this possibility, we compared the ability of TAP-deficient mammalian cells to express K^b-$OVA_{257–264}$ complexes. Following coinfection with VV-$ESOVA_{257–264}$ and VV-$K^b\beta_2$m at 28°C, T2 cells expressed only 1.5-fold as much Y3-reactive K^b as *Aedes* cells (Table II). By contrast, T2 cells expressed 30-fold more K^b-$OVA_{257–264}$ complexes than *Aedes* cells. In other experiments, T2 cells were up to 100-fold more effective at assembling endogenous K^b-$OVA_{257–264}$ complexes. This indicates that relative to mammalian cells, *Aedes* cells have a greatly reduced capacity for creating class I complexes from endogenously synthesized ER-targeted peptides.

Effect of tapasin expression on the production of K^b-$OVA_{257–264}$ complexes

Tapasin, which is necessary for association of TAP with class I molecules, binds to class I molecules before TAP association (11) and also appears to bind antigenic peptides (10). This raises the possibility that tapasin is needed for the loading of TAP-independent peptides. Tapasin appears to be a dedicated component of the class I Ag processing pathway, since it is induced by IFN-γ and, as such, is not expected to be expressed in insect cells. To investigate whether this could contribute to the poor assembly of K^b-$OVA_{257–264}$ complexes, we examined the capacity of .220 cells to assemble such complexes.

.220 cells are radiation-induced mutant EBV-transformed lymphocytes with a compromised capacity to assemble class I molecules, stemming from their absence of tapasin (11, 43, 44). .220

Table II. *Expression of endogenous K_b-$OVA_{257–264}$ complexes by human and* Aedes *cells*[a]

Cells	Expt.	MCF Y3	MCF 25-D1.16	% 25-D1.16 Reactive
T2	1	1712	1382	80.7
Aedes	1	1198	42	3.5
.45	2	528	409	77.4
	3	192	149	77.6
.220	2	331	148	44.7
	3	147	63	42.9
Aedes	2	128	4	3.1
	3	63	0	0

[a] Designated cells were infected for 14 h at 28°C with rVVs as indicated and the amount of cell surface K^b or K^b-$OVA_{257–264}$ complexes determined cytofluorographically using Y3 or 25-D1.16 mAb, respectively. Values represent mean channel fluorescence (MCF) and are not directly comparable between experiments due to different settings on the cytofluorograph. Background values of Ab binding to control VV-infected cells have been subtracted. These values are (in descending order according to the table) for Y3: 38, 59, 22, 12, 14, 12, 31, 37; and for 25-D1.16: 59, 60, 25, 17, 15, 13, 38, 40. The value in the last column was derived by dividing the values in the adjacent columns and multiplying by 100.

cells and control, tapasin-expressing .45 cells were infected overnight with the appropriate rVVs at 28°C, and the expression of K^b and K^b-$OVA_{257–264}$ complexes was determined by Y3 and 25-D1.16 staining, respectively (Table II). Despite the absence of tapasin, .220 cells were able to assemble class I complexes at ~55% the efficiency of .45 cells. By comparison, in the same experiments, the efficiency of complex assembly in *Aedes* cells was 0 to 3% of the efficiency of .45 cells. Given the potential differences between the levels of TAP-transported peptides in .45 and .220 cells that will compete with binding of $OVA_{257–264}$ to K^b, as well as the differences in the degrees of rVV coinfection of the two cell lines, we do not believe that the difference in assembly efficiency is significant. This conclusion is supported by the finding that the efficiency of complex formation in HeLa cells (described in the next section) is even slightly lower than in .220 cells.

To examine more directly the role of tapasin in the generation of K^b-$OVA_{257–264}$ complexes, we inserted the human tapasin gene into VV. Following infection of human cells, this rVV produced a protein of the correct M_r in SDS-PAGE that coprecipitated with human TAP, using a mAb specific for TAP1 (the biochemical characterization of VV-expressed tapasin will be described in a future publication). As seen in Table III, infection of .220/B8 cells with VV-tapasin enhanced the cell surface expression of endogenous class I molecules 1.7-fold, as determined using FITC-conjugated Abs specific for β_2m. By contrast, infection with VV-ICAM-1 resulted in an 0.7-fold decrease in β_2m expression. Such decreased class I cell surface expression is frequently associated with VV infection. As seen in Table III, tapasin coexpression with $K^b\beta_2$m and ES-$OVA_{257–264}$ had only a marginal effect on expression of Y3-reactive K^b molecules (11% enhancement) or K^b-$OVA_{257–264}$ complexes (14% enhancement) relative to a control rVV expressing influenza virus nucleoprotein (NP). These findings clearly demonstrate that tapasin is not required for the generation of K^b-$OVA_{257–264}$ complexes in VV-infected human cells, and it has has only a slight effect in enhancing complex generation.

We next examined the effect of tapasin expression on the K^b expression in *Aedes* cells (Table IV). Coinfection of cells with VV-tapasin had no effect on the generation of cell surface Y3-reactive K^b molecules and did not enhance the generation of K^b-$OVA_{257–264}$ complexes, which were undetectable in this experiment. This demonstrates that tapasin is not the sole factor preventing insect cells from assembling K^b-$OVA_{257–264}$ complexes

Table III. *Effect of tapasin expression on class I-peptide complex formation in tapasin deficient human cells*[a]

Virus	MCF			
	Anti-ICAM-1	Anti-β_2m	Y3	25-D1.16
Direct immunofluorescence				
ICAM-1	1521	285		
Tapasin	14	635		
None	8	383		
Indirect immunofluorescence				
$K^b\beta_2$m and $ESOVA_{257–264}$ and tapasin			487	291
$K^b\beta_2$m and $ESOVA_{257–264}$ and NP			431	249

[a] .220/B8 cells were infected for 14 h at 37°C with rVVs indicated and the amount of cell surface mouse ICAM-1, human β_2m, K^b, or K^b-$OVA_{257–264}$ complexes determined cytofluorographically by direct or indirect immunofluorescence. Direct immunofluorescence was performed using a FITC-conjugated mAb specific for ICAM-1 (PharMingen), and FITC-conjugated rabbit anti-β_2m Abs (Dako). Values represent mean channel fluorescence (MCF). Background values of Ab binding to control VV-infected cells have been subtracted; for Y3, 21; for 25-D1.16, 22.

with greater efficiency. Note, however, that it is plausible that other human gene products are required for the proper interaction of tapasin with class I molecules, a possibility that is presently under investigation.

Generation of endogenous K^b-$OVA_{257–264}$ complexes in insect cells: effects of protease inhibitors

Insect cells have not evolved with the requirement to present peptides to the immune system and may either possess proteases that rapidly destroy oligopeptides or lack chaperones to protect peptides from proteasomes or other proteases. To examine whether the $OVA_{257–264}$ peptide or its precursor is destroyed by cellular proteases, we determined the effects of four protease inhibitors on K^b-$OVA_{257–264}$ complex formation. cbz-LLL-CHO and cbz-LLF-CHO are peptide aldehydes that competitively inhibit proteasomes as well as other proteases (45). cbz-LL-CHO exhibits overlapping inhibition of cellular proteases with the tripeptide aldehydes, but does not block mammalian proteasomes at the concentrations used (46). Lactacystin is a microbial product that blocks proteasomes by covalently binding to the catalytic site (47). Inhibitors were used over a fourfold concentration range (Fig. 5). The effects of the inhibitors on complex generation were determined using the 25-D1.16 mAb to measure cell surface complexes. Effects of the inhibitors on K^b expression were determined using the Y3 mAb.

In this experiment, *Aedes* cells failed to produce K^b-$OVA_{257–264}$ complexes above background values obtained using the 25-D1.16 mAb against uninfected cells. It was particularly impressive, therefore, that complexes were easily detected in the presence of cbz-LeuLeu and, to a much lesser extent, cbz-LeuLeuLeu. Both inhibitors interfered with VV gene expression (this can be seen by the inhibition of K^b expression and was also observed for mouse CD54, influenza virus hemagglutinin, human CD4, and human CD26 (not shown)), which probably reduced the enhancing effects of these drugs on complex formation. Two findings indicate that these effects are not due to inhibition of blocking proteasome activity. First, neither cbz-LeuLeuLeu, a highly potent proteasome inhibitors, nor lactacystin, a highly specific proteasome inhibitor, had an effect on complex formation. Second, cbz-LeuLeu does not inhibit proteasomes at the concentrations used. cbz-LL-CHO is a potent inhibitor of calpains, but calpains are unlikely to be involved in peptide destruction, since we found no effect on expression of K^b-$OVA_{257–264}$ complex using calpain inhibitors I and II (not shown).

Using HeLa cells in the same experiment, we confirmed the efficient loading of K^b molecules with $OVA_{257–264}$ (as noted above, the value of 40% efficiency of loading is similar to that observed in .220 cells) and found that cbz-LL-CHO had only an inhibitory effect on expression of K^b-$OVA_{257–264}$ complexes, again probably related to its inhibition of viral gene expression (Fig. 5). In another experiment, cbz-LL-CHO failed to enhance formation of K^b-$OVA_{257–264}$ complexes in TAP-deficient human .174 cells (not shown). These data suggest that the effects of cbz-LL-CHO in insect cells are due to features of insect cells that are unfavorable to the generation or intracellular trafficking of antigenic peptides or their precursors.

cbz-LL-CHO could act either by enhancing the formation of complexes or by reducing destruction of cell surface complexes. To distinguish these possibilities, VV-$K^b\beta_2$m-infected *Aedes* cells were pulsed with peptide and incubated at 28°C for up to 3.5 h in the presence or absence of cbz-LL-CHO. cbz-LL-CHO did not affect the stability of cell surface K^b-$OVA_{257–264}$ complexes or Y3-reactive K^b molecules (Fig. 6), indicating that it acts by enhancing complex formation.

Table IV.[a]

Virus	MCF	
	Y3	25-D1.16
$K^b\beta_2$m and tapasin	288	2
$K^b\beta_2$m ICAM-1	283	0
$K^b\beta_2$m and ES-$OVA_{257–264}$ and tapasin	202	0
$K^b\beta_2$m and $ESOVA_{257–264}$ ICAM-1	212	0

[a] *Aedes* cells were infected for 14 h at 28°C with rVVs indicated and the amount of cell surface K^b or K^b-$OVA_{257–264}$ complexes determined cytofluorographically by indirect immunofluorescence. Values represent mean channel fluorescence (MCF). Background values of Ab binding to control VV-infected cells have been subtracted: for Y3, 68; for 25-D1.16, 63. The decrease in K^b expression observed in the bottom two rows is due to the competitive effects of the additional rVV.

Discussion

Our findings indicate that *Aedes* cells are capable of generating similar amounts of peptide-receptive cell surface rVV-encoded class I molecules as mammalian cells, but produce complexes from an endogenously produced ER-targeted peptide far less efficiently than mammalian cells. Due to the availability of the 25-D1.16 mAb, we have focused these studies on generation of the K^b-$OVA_{257–264}$ complexes. The generality of the our findings, however, are supported by additional experiments (unpublished) in which we failed to observe the generation of thermostabile K^d

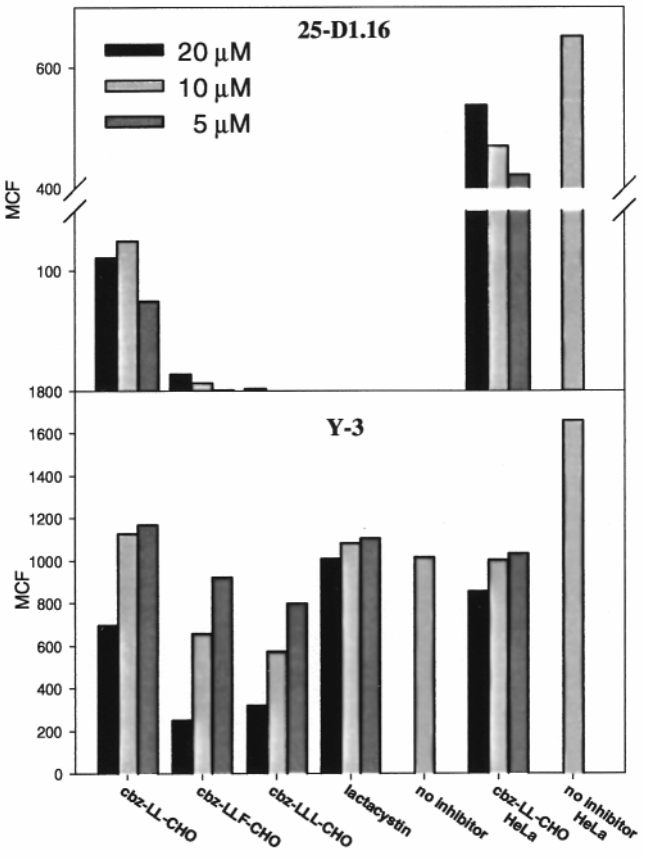

FIGURE 5. Effect of protease inhibitors on expression of cell surface K^b-$OVA_{257–264}$ complexes. The amounts of K^b molecules or K^b-$OVA_{257–264}$ complexes present on the surface of viable *Aedes* cells (first five sets of columns) or HeLa cells infected with VV-$K^b\beta_2$m and VV-$ESOVA_{257–264}$ in the presence of protease inhibitors at the indicated concentrations was determined by three-step immunofluorescence using 25-D1.16 and Y3 mAbs. Background staining values against uninfected *Aedes* and HeLa cells are subtracted; these are, respectively, 25-D1.16, 49 and 20; Y3, 86 and 20.

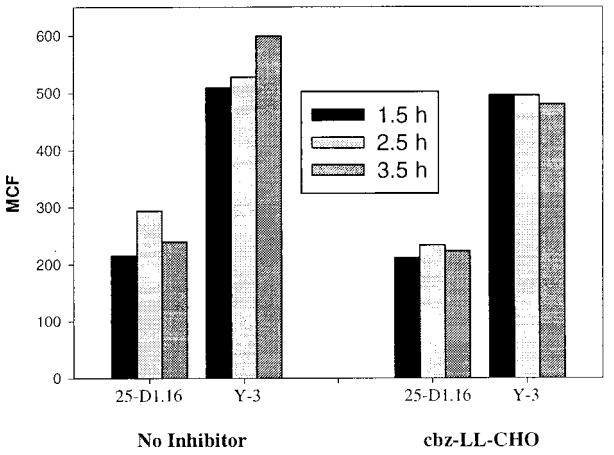

FIGURE 6. Effect of cbz-LL-CHO on stability of K^b-$OVA_{257–264}$ complexes. *Aedes* cells infected with VV-$K^b\beta_2$m and incubated with $OVA_{257–264}$ synthetic peptide to create K^b-$OVA_{257–264}$ complexes were incubated for the indicated times at 28°C in the presence or absence of 10 μM cbz-LL-CHO. The amounts of K^b molecules or K^b-$OVA_{257–264}$ complexes present on the surface of viable *Aedes* cells infected with VV-$K^b\beta_2$m and VV-$ESOVA_{257–264}$ were determined by standard two-step immunofluorescence using Y3 and 25-D1.16 mAbs, respectively.

molecules following the infection of *Aedes* cells with a rVV co-expressing K^d with mouse β_2m and a rVV expressing an ER-targeted, K^d-binding peptide (corresponding to residues 147 to 155 from influenza virus NP). We previously showed that coinfection of T2 cells with these rVVs results in expression of considerable numbers of thermostabile, cell surface K^d molecules (48).

The rapidity of class I assembly and export from the ER in *Aedes* cells (Fig. 2), which is similar to that observed in mammalian cells, contrasts to the findings obtained with *Drosophila* cells, where Y3-reactive K^b export from the ER occurred two to three times more slowly than mammalian cells (17). Expression of canine calnexin in *Drosophila* cells slowed ER export even further (17), although it had the salubrious effect of enhancing the efficiency of assembly (49). We have not examined the efficiency of K^b folding in *Aedes* cells, and it is possible that it is low relative to mammalian cells. Following coinfection of *Aedes* cells with a rVV-expressing canine calnexin, however, we have failed to detect any difference in K^b folding, trafficking, cell surface expression, or ability to assemble with ER-targeted $OVA_{257–264}$ (unpublished findings). It is plausible that *Aedes* cells express ER chaperones that are better able to facilitate K^b folding than those present in *Drosophila* cells. Another possibility is that *Drosophila* chaperones are fully able to support K^b folding, but are present in limiting quantities and cannot cope with the possibly large amount of K^b produced from a strong promoter. Findings that class I molecules are efficiently assembled in mutant cells lacking either calnexin (9) or a glucosidase needed for calnexin function (8) indicate that calnexin is not required for assembly of class I molecules. More directly, we recently demonstrated that calnexin is not required for the assembly of K^b-$OVA_{257–264}$ complexes (50). In any event, insect cells express both calnexin and calreticulin homologues (51, 52). Given that calnexin interacts with oligosaccharides present in all eukaryotic cells and its ability to interact with numerous proteins, it seems likely that insect calnexin/calreticulin can interact with class I α-chains.

The major finding in this study is that TAP-independent assembly of K^b molecules with an ER-targeted peptide occurs inefficiently in *Aedes* cells relative to mammalian cells and can be enhanced by protease inhibitors. There are several plausible mechanisms that could contribute to this finding.

1. The delivery of $ESOVA_{257–264}$ to the ER may be less efficient in insect cells than in mammalian cells. This could be due to either decreased delivery to the ER from the cytosol or inefficient liberation of the peptide from the signal sequence by signal peptidase. The latter possibility is unlikely, since first, mammalian and insect signal peptidases are not known to differ in specificity; and second, $ESNP_{147–155}$ is similarly inept at providing peptides capable of associating with K^d in the ER despite having a different junctional sequence. The former possibility cannot be as easily dismissed, and indeed, cbz-LL-CHO might enhance the generation of K^b-$OVA_{257–264}$ complexes by prolonging the survival of $ESOVA_{257–264}$ in the cytosol. Notably, $ESOVA_{257–264}$ cannot be targeted cotranslationally to the ER by signal recognition particle, since its limited size (26 residues) precludes emergence of the signal sequence from the ribosome before translation termination (this requires ~40 residues). Thus, $ESOVA_{257–264}$ is probably exposed to cytosolic proteases before its translocation, and it is plausible that *Aedes* cells more actively degrade cytosolic peptides than mammalian cells, due either to enhanced proteolysis or diminished chaperone-mediated protection (53).

2. Evolution may have altered the protease activity of the ER of vertebrate cells to minimize the destruction of class I binding peptides, while optimizing trimming of TAP-transported peptides, particularly those with NH_2-terminal extensions (54).

3. The ER of mammalian cells may contain dedicated chaperones that protect antigenic peptides from proteolysis and ferry them to class I molecules. There would be a number of candidates for such an ER-localized peptide chaperone. By virtue of its ability to bind both class I molecules and peptides, tapasin was the prime candidate for the missing chaperone in insect cells. Our findings clearly indicate, however, that tapasin is dispensable for the formation of K^b-$OVA_{257–264}$ complexes in human lymphoid cells. Srivastava and colleagues have shown that gp96, a resident ER chaperone, binds antigenic peptides and has the capacity to provide them to class I molecules for immune recognition (55). Other mammalian chaperones are known to bind antigenic peptides (53); indeed, TAP-transported peptides bind to numerous ER chaperones (56, 57). Chaperones are highly conserved among eukaryotes, and insect cells possess close homologues for all of the mammalian chaperones reported to bind antigenic peptides. Since antigenic peptides probably bind to regions in chaperones that recognize unfolded proteins, it is likely that insect chaperones bind antigenic peptides to an extent similar to mammalian chaperones. One or more vertebrate chaperones, however, may possess alterations that enable them to participate in Ag processing in a more directed manner than chaperones from lower eukaryotes.

In future studies, it should be possible to use the system we describe herein to systematically define the specialized components that enable mammalian cells to efficiently generate peptide-class I complexes in the ER.

Acknowledgments

Bethany Buschling provided outstanding technical assistance. We thank Nilabh Shastri (University of California, Berkeley) for providing the B3Z cells, Marian Orlowski (Mt. Sinai School of Medicine, New York, NY) for the panel of protease inhibitors, Thomas Spies (Fred Hutchinson Cancer Institute, Seattle, WA) for .220 cells, and Robert DeMars (University of Wisconsin, Madison, WI) for .45 cells.

References

1. Townsend, A., and H. Bodmer. 1989. Antigen recognition by class I-restricted T lymphocytes. *Annu. Rev. Immunol. 7:601.*
2. Yewdell, J.W., and J. R. Bennink. 1992. Cell biology of antigen processing and presentation to MHC class I molecule-restricted T lymphocytes. *Adv. Immunol. 52:1.*
3. Rammensee, H.-G., T. Friede, and S. Stevanovic. 1995. MHC ligands and peptide motifs: first listing. *Immunogenetics 41:178.*
4. Zinkernagel, R .M., and P. C. Doherty. 1979. MHC-restricted cytotoxic T cells: studies on the biological role of polymorphic major transplantation antigens determining T-cell restriction-specificity, function, and responsiveness. *Adv. Immunol. 27:52.*
5. Degen, E., and D. B. Williams. 1991. Participation of a novel 88-kDa protein in the biogenesis of murine class I histocompatibility molecules. *J. Cell Biol. 112: 1099.*
6. Hochstenbach, F., V. David, S. Watkins, and M. B. Brenner. 1992. Endoplasmic reticulum resident protein of 90 kilodaltons associates with the T- and B-cell antigen receptors and major histocompatibility complex antigens during their assembly. *Proc. Natl. Acad. Sci. USA 89:4734.*
7. Nöβner, E., and P. Parham. 1995. Species-specific differences in chaperone interaction of human and mouse major histocompatibility complex class I molecules. *J. Exp. Med. 181:327.*
8. Balow, J. P., J. D. Weissman, and K. P. Kearse. 1995. Unique expression of major histocompatibility complex class I proteins in the absence of glucose trimming and complex association. *J. Biol. Chem. 270:29025.*
9. Scott, J. E., and J. R. Dawson. 1995. MHC class I expression and transport in a calnexin-deficient cell line. *J. Immunol. 155:143.*
10. Li, S., H. O. Sjogren, U. Hellman, R. F. Pettersson, and P. Wang. 1997. Cloning and functional characterization of a subunit of the transporter associated with antigen processing. *Proc. Natl. Acad. Sci. USA 94:8708.*
11. Sadasivan, B., P. J. Lehner, B. Ortmann, T. Spies, and P. Cresswell. 1996. Roles of calreticulin and novel glycoprotein, tapasin, in the interaction of MHC class I molecules with TAP. *Immunity 5:103.*
12. Suh, W.-K., E. K. Mitchell, Y. Yang, P. A. Peterson, G. L. Waneck, and D. B. Williams. 1996. MHC Class I molecules form ternary complexes with calnexin and TAP and undergo peptide-regulated interaction with TAP via their extracellular domains. *J. Exp. Med. 184:337.*
13. Heemels, M.-T., and H. Ploegh. 1995. Generation, translocation, and presentation of MHC class-I restricted peptides. *Annu. Rev. Biochem. 64:463.*
14. Ortmann, B., M. J. Androlewicz, and P. Cresswell. 1994. MHC class I/β_2-microglobulin complexes associate with TAP transporters before peptide binding. *Nature 368:864.*
15. 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 264:1322.*
16. Jackson, M. R., E. S. Song, Y. Yang, and P. A. Peterson. 1992. Empty and peptide-containing conformers of class I major histocompatibility complex molecules expressed in *Drosophila melanogaster* cells. *Proc. Natl. Acad. Sci. USA 89:12117.*
17. Jackson, M. R., M. F. Cohen-Doyle, P. A. Peterson, and D. B. Williams. 1994. Regulation of MHC class I transport by the molecular chaperone, calnexin (p88, IP90). *Science 263:384.*
18. Lévy, F., and S. Kvist. 1991. Co-expression of the human HLA-B27 class I antigen and the E3/19K protein of adenovirus-2 in insect cells using a baculovirus vector. *Int. Immunol. 2:995.*
19. Godeau, F., I. F. Luescher, D. M. Ojcius, C. Saucier, E. Mottez, L. Cabanie, and P. Kourilsky. 1992. Purification and ligand binding of a soluble class I major histocompatibility complex molecule consisting of the first three domains of H-$2K^d$ fused to β_2-microglobulin expressed in the baculovirus-insect cell system. *J. Biol. Chem. 267:24223.*
20. Meyer, T. H., P.M. van Endert, S. Uebel, B. Ehring, and R. Tampé. 1994. Functional expression and purification of the ABC transporter complex associated with antigen processing (TAP) in insect cells. *FEBS Lett. 351:443.*
21. Bennink, J. R., and J. W. Yewdell. 1990. Recombinant vaccinia viruses as vectors for studying T lymphocyte specificity and function. *Curr. Top. Microbiol. Immunol. 163:153.*
22. Karttunen, J., and N. Shastri. 1991. Measurement of ligand-induced activation in single viable T cells using the *lacZ* reporter gene. *Proc. Natl. Acad. Sci. USA 88:3972.*
23. Cox, J. H., J. W. Yewdell, L. C. Eisenlohr, P. R. Johnson, and J. R. Bennink. 199 Antigen presentation requires transport of MHC class I molecules from the endoplasmic reticulum. *Science 247:715.*
24. Bacik, I., J. H. Cox, R. Anderson, J. W. Yewdell, and J. R. Bennink. 1994. TAP-independent presentation of endogenously synthesized peptides is enhanced by endoplasmic reticulum insertion sequences located at the amino but not carboxy terminus of the peptide. *J. Immunol. 152:381.*
25. Coupar, B. E. H., M. E. Andrew, and D. B. Boyle. 1988. A general method for the construction of recombinant vaccinia viruses expressing multiple foreign genes. *Gene 68:1.*
26. Russ, G., F. Esquivel, P. Cresswell, T. Spies, J. W. Yewdell, and J. R. Bennink. 1995. Assembly, intracellular localization, and nucleotide binding properties of the human peptide transporters Tap1 and Tap2 expressed by recombinant vaccinia viruses. *J. Biol. Chem. 270:21312.*
27. Franke, C.A., and D. E. Hruby. 1985. Expression of recombinant vaccinia virus-derived alphavirus proteins in mosquito cells. *J. Gen. Virol. 66:2761.*
28. Ortiz-Navarrete, V., and G. J. Hämmerling. 1991. Surface appearance and instability of empty H-2 class I molecules under physiological conditions. *Proc. Natl. Acad. Sci. USA 88:3594.*
29. Ljunggren, H.-G., N.J. Stam, C. Öhlén, J. J. Neefjes, P. Höglund, E. Heinen, J. Bastin, T. N. M. Schumacher, A. Townsend, K. Kärre, and H. L. Ploegh. 1990. Empty MHC class I molecules come out in the cold. *Nature 346:476.*
30. Potter, T. A., R. A. Zeff, A.-M. Schmitt-Verhulst, and T. V. Rajan. 1985. Molecular analysis of an EL4 cell line that expresses H-$2D^b$ but no H-$2K^b$ or β_2-microglobulin. *Proc. Natl. Acad. Sci. USA 82:2950.*
31. Allen, H., J. Fraser, D. Flyer, S. Calvin, and R. Flavell. 1986. β_2-microglobulin is not required for cell surface expression of the murine class I histocompatibility antigen H-$2D^b$ or of a truncated H-$2D^b$. *Proc. Natl. Acad. Sci. USA 83:7447.*
32. Koller, B. H., P. Marrack, J. W. Kappler, and O. Smithies. 1990. Normal development of mice deficient in β_2-microglobulin, MHC class I proteins, and CD^+ T cells. *Science 248:1227.*
33. Williams, D. B., B. H. Barber, R. A. Flavell, and H. Allen. 1989. Role of β_2-microglobulin in the intracellular transport and surface expression of murine class I histocompatibility molecules. *J. Immunol. 142:2796.*
34. Song, E. S., Y. Yang, M. R. Jackson, and P. A. Peterson. 1994. In vivo regulation of the assembly and intracellular transport of class I major histocompatibility complex molecules. *J. Biol. Chem. 269:7024.*
35. Degen, E., M. F. Cohen-Doyle, and D. B. Williams. 1992. Efficient dissociation of the p88 chaperone from major histocompatibility complex class I molecules requires both β_2-microglobulin and peptide. *J. Exp. Med. 175:1653.*
36. Falk, K., O. Rötzschke, S. Stevanovic, G. Jung, and H.-G. Rammensee. 1991. Allele-specific motifs revealed by sequencing of self-peptides eluted from MHC molecules. *Nature 351:290.*
37. Carbone, F. R., M. W. Moore, J. M. Sheil, and M. J. Bevan. 1988. Induction of CTL by primary in vitro stimulation with peptides. *J. Exp. Med. 167:1767.*
38. Khilko, S. N., M. Corr, L. F. Boyd, A. Lees, J. K. Inman, and D. H. Margulies. 1993. Direct detection of major histocompatibility complex class I binding to antigenic peptides using surface plasmon resonance: peptide immobilization and characterization of binding specificity. *J. Biol. Chem. 268:15425.*

39. Day, P.M., J. W. Yewdell, A. Porgador, R. N. Germain, and J. R. Bennink. 1997. Direct delivery of exogenous MHC class I molecule-binding oligopeptides to the endoplasmic reticulum of viable cells. *Proc. Natl. Acad. Sci. USA 94:8064.*
40. Cai, Z., A. Brunmark, M. R. Jackson, D. Loh, P. A. Peterson, and J. Sprent. 1996. Transfected Drosophila cells as a probe for defining the minimal requirements for stimulating unprimed $CD8^+$ T cells. *Proc. Natl. Acad. Sci. USA 93:14736.*
41. 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.*
42. Porgador, A., J. W. Yewdell, Y. Deng, J. R. Bennink, and R. N. Germain. 1997. Localization, quantitation, and in situ detection of specific peptide-MHC class I complexes using a mAb. *Immunity 6:715.*
43. Grandea, A. G., III, M. J. Androlewicz, R. S. Athwal, D. E. Geraghty, and T. Spies. 1995. Dependence of peptide binding by MHC class I molecules on their interaction with TAP. *Science 270:105.*
44. Ortmann, B., J. Copeman, P. J. Lehner, B. Sadasivan, J. A. Herberg, A. G. Grandea, S. R. Riddell, R. Tampé, T. Spies, J. Trowsdale, and P. Cresswell. 1997. A critical role for tapasin in the assembly and function of multimeric MHC class I-TAP complexes. *Science 277:1306.*
45. Vinitsky, A., C. Cardozo, L. Sepp-Lorenzino, C. Michaud, and M. Orlowski. 1994. Inhibition of the proteolytic activity of the multicatalytic proteinase complex (proteasome) by substrate-related peptidyl aldehydes. *J. Biol. Chem. 269: 29860.*
46. Vinitsky, A., L. C. Antón, H. L. Snyder, M. Orlowski, J. R. Bennink, and J. W. Yewdell. 1997. The generation of MHC class I-associated peptides is only partially inhibited by proteasome inhibitors: involvement of nonproteasomal cytosolic proteases in antigen processing? *J. Immunol. 159:554.*
47. Fenteany, G., R. F. Standaert, W. S. Lane, S. Choi, E. J. Corey, and S. L. Schreiber. 1995. Inhibition of proteasome activities and subunit-specific amino-terminal threonine modification by lactacystin. *Science 268:726.*
48. Deng, Y., J. W. Yewdell, L. C. Eisenlohr, and J. R. Bennink. 1997. MHC affinity, peptide liberation, T cell repertoire, and immunodominance all contribute to the paucity of MHC class I-restricted peptides recognized by antiviral CTL. *J. Immunol. 158:1507.*
49. Vassilakos, A., M. F. Cohen-Doyle, P. A. Peterson, M. R. Jackson, and D. B. Williams. 1996. The molecular chaperone calnexin facilitates folding and assembly of class I histocompatibility molecules. *EMBO J. 15:1495.*
50. Prasad, S. A., J.W. Yewdel., A. Porgador, B. Sadasivan, P. Cresswell, and J. R. Bennink. 1998, Calnexin expression does not enhance the generation of MHC class I-peptide complexes. *Eur. J. Immunol. 28:907.*
51. Christodoulou, S., A. E. Lockyer, J.M. Foster, J. D. Hoheisel, and D.B. Roberts. 1997. Nucleotide sequence of a *Drosophila melanogaster* cDNA encoding a calnexin homologue. *Gene 191:143.*
52. Smith, M.J. 1992. Nucleotide sequence of a *Drosophila melanogaster* gene encoding a calreticulin homologue. *DNA Seq. 3:247.*
53. Udono, H., and P. K. Srivastava. 1993. Heat shock protein 70-associated peptides elicit specific cancer immunity. *J. Exp. Med. 178:1391.*
54. Snyder, H.L., J. W. Yewdell, and J. R. Bennink. 1994. Trimming of antigenic peptides in an early secretory compartment. *J. Exp. Med. 180:2389.*
55. Udono, H., D. L. Levey, and P. K. Srivastava. 1994. Cellular requirements for tumor-specific immunity elicited by heat shock proteins: Tumor rejection antigen gp96 primes $CD8^+$ T cells in vivo. *Proc. Natl. Acad. Sci. USA 91:3077.*
56. Lammert. E., S. Stevanovic, J. Brunner, H. G. Rammensee, and H. Schild. 1997. Protein disulfide isomerase is the dominant acceptor for peptides translocated into the endoplasmic reticulum. *Eur. J. Biochem. 27:1685.*
57. Marusina, K., G. Reid, R. Gabathuler, W. Jefferies, and J. J. Monaco. 1997. Novel peptide-binding proteins and peptide transport in normal and TAP-deficient microsomes. *Biochemistry 36:856.*