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Immunology

Direct delivery of exogenous MHC class I molecule-binding oligopeptides to the endoplasmic reticulum of viable cells

PATRICIA M. DAY*, JONATHAN W. YEWDELL*†, ANGEL PORGADOR*‡, RONALD N. GERMAIN*‡, AND JACK R. BENNINK*†

Laboratories of *Viral Diseases and ‡Immunology, National Institute of Allergy and Infectious Diseases, Bethesda, MD 20892-0440

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ABSTRACT **After brief incubation of cells with fluorescein-conjugated peptides that bind major histocompatibility complex (MHC) class I molecules, peptides were detected within the endoplasmic reticulum (ER) by microscopy or by binding to radiolabeled class I molecules. ER delivery of a nonfluorescent peptide was demonstrated using a mAb highly specific for the peptide–class I molecule complex. ER localization of peptides: (*i*) required expression of appropriate class I molecules in the ER but not on the cell surface, (*ii*) was diminished by expression of TAP, the MHC-encoded cytosol to ER peptide transporter, and (*iii*) was blocked by pinocytosis inhibitors but not by brefeldin A. These findings demonstrate the existence of a pathway, likely vesicular in nature, that conveys small extracellular substances to the ER without traversing the Golgi complex or the cytosol. This pathway contributes to the loading of exogenous peptides to MHC class I molecules, but its evolutionary significance may lie in other cellular processes, such as maintaining ER homeostasis or signaling by extracellular substances.**

Major histocompatibility complex (MHC) class I molecules consist of an integral membrane glycoprotein (α chain) noncovalently complexed to a smaller soluble subunit (β_2-microglobulin). Class I molecules function to bind peptides of 8–10 residues and present them at the cell surface to $CD8^+$ T cells, enabling immune surveillance of intracellular proteins. Most class I-associated peptides are produced by proteases located in the cytosol and are transported into the endoplasmic reticulum (ER) through the action of TAP, the MHC-encoded peptide transporter (1, 2). The direct association of TAP with class I molecules and additional chaperones (3, 4) greatly facilitates peptide loading of class I molecules.

TAP association with class I molecules is not, however, essential for loading of peptides. Peptides targeted to the ER via the translocon associate with class I molecules in TAP-deficient cells (5–7). Culturing TAP-deficient cells in the presence of class I-binding synthetic peptides bolsters class I surface expression and the amount of [^{35}S]-methionine-radiolabeled class I molecules recovered from detergent extracts. It was originally proposed that this was caused by peptides reaching the site of class I assembly in the ER (8). Subsequently, it was demonstrated that pinocytosed peptide released upon detergent extraction contributed at least partially to the increased recovery of [^{35}S]-methionine-labeled class I molecules (9). Moreover, it was later demonstrated that exogenous peptides bind to class I molecules present on the cell surface, and indeed that most "peptide receptive" molecules are created by the binding of β_2-microglobulin present in the culture medium to free α chains on the cell surface (10). These findings did not, however, conclusively eliminate the possibility of direct trafficking of exogenous peptides to the cell surface. In the present study we have reexamined this question.

MATERIALS AND METHODS

Cell Lines. RMA and RMA/S cells (11) were maintained in air/CO_2 (94%/6%) in RPMI 1640 medium supplemented with 7.5% (vol/vol) fetal bovine serum. L929 and LK^b cells were maintained in air/CO_2 (91%/9%) in DMEM supplemented with 7.5% (vol/vol) fetal bovine serum. T2 cells (12) were maintained in air/CO_2 (91%/9%) in Iscove's modified DMEM supplemented with 7.5% (vol/vol) fetal bovine serum.

Synthetic Peptides. Fluorescein isothiocyanate-conjugated and nonconjugated synthetic peptides were obtained from the Laboratory of Molecular Structure, National Institute of Allergy and Infectious Diseases. Peptides were produced, purified, and characterized as described (13).

Peptide Localization in Cells. L929 or LK^b cells were cultured overnight on 12-mm, acid-cleaned #1 glass coverslips in 24-well tissue culture plates. K^bFL was added directly to cells in growth medium and was incubated for 15 min at 37°C in air/CO_2 (91%/9%). After thorough rinsing with PBS, coverslips were inverted onto 7 μl of glycerol/PBS (70% vol/vol, pH 8.0), sealed with nail polish, and immediately examined by confocal microscopy. For intracellular staining with fluorescein-labeled 25-D1.16 mAb, cells were fixed with 1% paraformaldehyde for 10 min at room temperature and permeabilized by inclusion of 0.1% Brij 58 (wt/vol) with the antibody. [The production and characterization of the 25-D1.16 mAb will be described in another publication (43).] Cells were stained for 15 min at 4°C, washed, and mounted with Fluoromount G (Southern Biotechnology Associates). For AlF_3, NaF was added to a final concentration of 30 mM, and $AlCl_3$ was added to a final concentration of 50 μm. Images were collected with a Bio-Rad MRC600 or MRC 1024 LSCM in the photon counting mode using a Zeiss Axioplan microscope and a ×40 (numerical aperture 1.3) plan-neofluor objective. The brightest sample in each experiment was used to accumulate frames until gray scale saturation was achieved; this number of frames then was accumulated for all of the specimens obtained in the same experimental protocol. Images were collated using ADOBE PHOTOSHOP software (Adobe Systems, Mountain View, CA) and printed with a FUJIX (Tokyo) Pictrography 3000 digital printer. For double staining of RMA/S cells, cells were incubated in suspension at 5×10^6 cells/ml with 10 μg/ml peptide for 2 h at 37°C. Cells were then washed thoroughly in PBS and fixed by incubation with 1% paraformaldehyde/PBS (wt/vol) for 10 min at room temperature and then washed with

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Abbreviations: MHC, major histocompatibility complex; ER, endoplasmic reticulum; TAP, transporter associated with antigen processing; BFA, brefeldin A; rVV, recombinant vaccinia virus; γIFN, γ-interferon.

†To whom reprint requests should be addressed. e-mail: jbennink@nih.gov; jyewdell@nih.gov.

PBS supplemented with 200 mM glycine. Binding of Con A to the cell surface was blocked by incubating fixed cells with 10 μg/ml unconjugated Con A for 10 min on ice. Cells were washed and permeabilized by inclusion of 0.1% Brij 58 (wt/vol) in all subsequent manipulations. Hybridoma tissue culture supernatant was used for the 25D1.16 staining. The secondary antibody was Texas Red conjugated donkey anti-mouse IgG (Jackson ImmunoResearch). Con A–fluorescein isothiocyanate (2.5 μg/ml) was included with the secondary antibody incubation. After staining, cells were resuspended in 8 μl of Fluoromount G and coverslipped. Images were collected as described above.

Cytofluorographic Quantitation of Peptide Binding. L cells were infected as described with recombinant vaccinia viruses (rVVs) (14). Four hours after infection, 1×10^6 cells were incubated for 60 min at 37°C with 5 μg/ml peptide in 150 μl of DMEM. Cells were then washed three times with PBS supplemented with 10 μM of sodium azide and analyzed using a cytofluorograph. For competition analyses, cells were preincubated with unlabeled peptide at 50 μg/ml for 10 min at 37°C before addition of labeled peptide. The competitor peptides and their corresponding, restricting class I molecules were VSV 52–59 (RGYVYQGL, K^b), NP 50–57 (SDYEGRLI, K^k), OVA 257–264 (SIINFEKL, K^b), and PR8-HA2 518–526 (IYSTVASSL, K^d). Indirect immunofluorescence was performed using fluorescein-conjugated rabbit anti-mouse IgG. Cells were fixed with 1% paraformaldehyde and permeabilized by including saponin (0.5%) in all solutions after fixation.

Biochemical Procedures. RMA or LK^b cells were cultured for 20 h in the presence of 20 units/ml γ-interferon (γIFN). After washing in PBS, cells were suspended in methionine-free DMEM and divided into four aliquots. Two samples were supplemented with K^bFL at 10 μg/ml. After a 30-min incubation, 300 μCi of [^{35}S]methionine was added to all aliquots except one of the peptide-incubated samples. After a 5-min incubation at 37°C, half of the cells were removed to ice, and the remainder incubated for a further 15 min at 37°C in the presence of 1 mM methionine. Aliquots then were washed extensively. The pulse and chase of the unlabeled, peptide-incubated cells were mixed with radiolabeled cells not exposed to peptide. Cells were then lysed on ice in extraction buffer (0.15 M NaCl/10 mM Tris, pH 7.4/1% (vol/vol) TX100/1 mM phenylmethylsulfonyl fluoride) containing 10 μg/ml SIINFEKL. After centrifugation for 10 min at $15{,}000 \times g$, the amounts of trichloroacetic acid-precipitable counts in the lysates were determined. Equal amounts of trichloroacetic acid-precipitable radioactivity were incubated with protein A–agarose beads preloaded with a rabbit antiserum specific for fluorescein or an antibody raised to a synthetic peptide corresponding to exon 8 of K^b (15). After washing, radioactive material was analyzed by SDS/PAGE (16). Gels were dried, and the radioactivity was visualized using a PhosphorImager (Molecular Dynamics). A similar protocol was used for T2 cells.

RESULTS

Localization of Fluorescein-Conjugated Peptides to the ER. To study the trafficking of synthetic peptides in live cells, we used two peptides that bind to H-2 K^d class I molecules, SYIPSAEKI and KYQAVTTTL, and a H-2K^b-binding peptide, SIINFEKL. Visualization and biochemical recovery were enabled by conjugating fluorescein to the ε-amino group of the lysine residue of each of the peptides during synthesis (the peptides are termed, respectively, "K^dFL1," "K^dFL2," and "K^bFL"). As described for K^bFL (13), conjugation of fluorescein did not alter the class I binding properties of the K^d-binding peptides as assessed by incubation with live cells on ice.

We initially observed by laser scanning confocal microscopy that incubation of L929 cells (L) or L cells expressing a K^b-transgene (LK^b) for 15 min at 37°C with K^bFL resulted in the equivalent accumulation of peptide in endosomal vesicles of both cells (Fig. 1*A*, *Left*; vesicles are the small intensely stained structures). This was expected because the cells are actively pinocytosing at 37°C. However, LK^b demonstrated less intense, but still clear, reticular staining of the cytoplasm and staining of the nuclear membrane characteristic of the ER of these cells. After removal of extracellular peptide and further incubation for 90 min at 37°C, the endosomal staining of both cell types was diminished whereas the specific reticular and nuclear membrane staining in LK^b cells persisted (Fig. 1*A*, *Right*). Reticular and nuclear membrane staining of L cells by K^bFl was much weaker than that of LK^b cells immediately after removal of peptide and rapidly fell to background levels upon further incubation at 37°C. After aldehyde fixation and detergent permeabilization of cells pulsed with K^bFL, fluorescence was no longer detected in L cells and was detected only in the ER of LK^b cells (not shown). This demonstrates that aldehyde fixation of the peptide requires binding to class I molecules. We interpret the loss of vesicular staining in LK^b cells upon

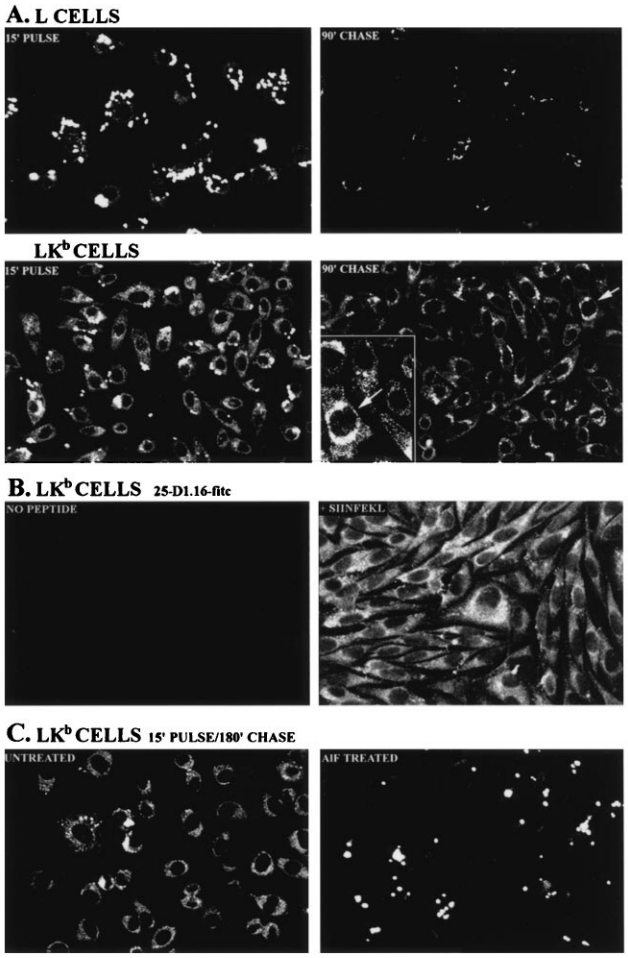

FIG. 1. Internalization of peptides in L and LK^b cells. (*A*) L or LK^b cells were exposed (*Left*) to the K^bFL peptide for 15 min and examined by laser scanning confocal microscopy or incubated (*Right*) for an additional 90 min at 37°C after removal of peptide before examination. (*Lower Right*, *Inset*) Enlargement of the region around the cell marked with an arrow to better observe the reticular staining pattern in the cytoplasm. (*B*) LK^b cells were treated with both 40 units/ml γIFN and 1 μg/ml BFA/ml for 2 h and 10 μM cbz-LeuLeuLeu (generously provided by M. Orlowski, Mt. Sinai School of Medicine, New York) for 1 h before addition of 10 μg/ml unmodified SIINFEKL peptide for 1 h. Cells were stained with fluorescein conjugated-25-D1.16. (*C*) LK^b cells were incubated for 15 min with K^bFL and then for 180 min at 37°C in the absence (*Left*) or presence (*Right*) of aluminum fluoride.

fixation and permeabilization to mean that peptides are not bound to class I molecules in endosomes.

Localization of Nonconjugated Peptides to the ER Using a mAb Specific for the Peptide–Class I Complex. The intracellular delivery of peptides was characterized further using a mAb, 25-D1.16. 25-D1.16 was produced by standard hybridoma technology from mice immunized with TAP-deficient cells expressing K^b molecules loaded with SIINFEKL. Binding of 25-D1.16 to tissue culture cells requires expression of K^b and either exposure to exogenous SIINFEKL or expression of proteins containing the SIINFEKL peptide (43). Using 25-D1.16, we could determine whether unconjugated SIINFEKL was delivered to the ER in a similar manner as K^bFL.

Live cells incubated with SIINFEKL were fixed, permeabilized, and stained with 25-D1.16 conjugated with fluorescein (Figs. 1*B* and 2). As expected, binding of 25-D1.16 required expression of K^b and exposure to peptide. Vesicular staining of K^b-expressing cells exposed to peptide was not detected, confirming that peptide present in vesicles represents pinocytosed material not bound to K^b. ER staining was preserved, however, as demonstrated by the characteristic reticular and nuclear membrane pattern of cytoplasmic staining. The generation of K^b–peptide complexes did not occur post-fixation because we were not able to create 25-D1.16-reactive K^b molecules by adding even high concentrations of peptides (5 μg/ml) after fixation. These findings were repeated with RMA/S cells (TAP-deficient mutants of RMA mouse lymphoma cells), in which we could also show that 25-D1.16 staining colocalized with fluorescein-conjugated Con A, whose binding serves as a marker for the ER because of its high affinity for the simple oligosaccharides characteristic of ER glycoproteins (Fig. 2). Note particularly the absolutely clear staining of the nuclear membrane in Fig. 2, which is a subdomain of the ER.

The cells shown in Figs. 1*B* and 2 were treated with brefeldin A (BFA), cbz-LeuLeuLeu, and γIFN before exposure to peptides to enhance peptide localization to the ER. In additional experiments, we determined that each of these compounds acting alone enhances the ER localization of exogenous peptides in LK^b cells.

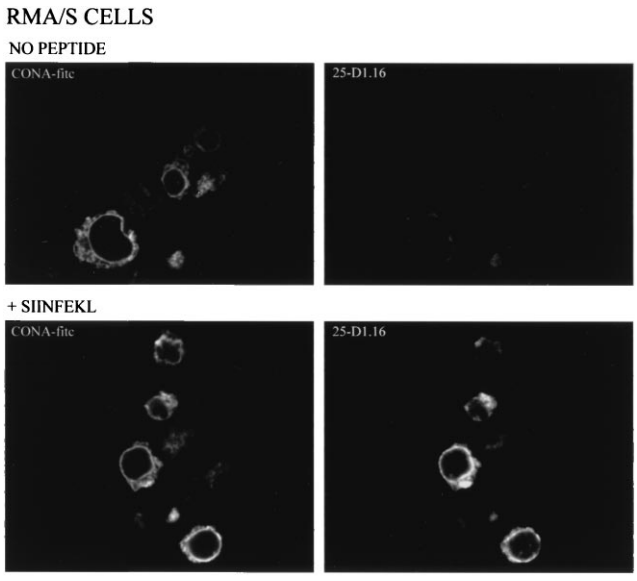

FIG. 2. Internalization of peptides in RMA/S cells. RMA/S cells were treated with 20 units of γIFN for 20 h and 1 μg/ml brefeldin A (BFA) for 3 h before peptide addition. Cells were then incubated with either the SIINFEKL peptide or no peptide for an additional 2 h. Cells were then fixed, permeabilized, and stained with the 25-D1.16 antibody detected with Texas Red conjugated donkey anti-mouse IgG (*Right*). Cells were coincidentally stained with fluorescein-conjugated Con A (*Left*).

This is consistent with the known effects of the compounds on class I biogenesis. BFA blocks transport of class I molecules from the ER (17, 18) and likely enhances the number of peptide-receptive class I molecules by retaining class I molecules with low affinity ligands. cbz-LeuLeuLeu inhibits many of the proteolytic activities of the proteasome (19) and presumably enhances staining by reducing the supply of class I binding peptides, thereby increasing the amount of peptide-receptive class I molecules in the ER. γIFN enhances class I biosynthesis and has been shown to augment the pool of peptide-receptive class I molecules in the ER (13).

Delivery of Exogenous Peptides to Class I Molecules Retained in the ER. We next quantitated the delivery of peptide to the ER by infecting L cells with an rVV expressing a genetically modified H-$2K^d$ molecule retained in the ER (termed "$EC15K^d$") by exchanging the cytosolic domain for that of the adenovirus E3/19K glycoprotein (20). K^dFL1 or K^dFL2 was incubated with cells infected with VV-$EC15K^d$, VV-K^d, or VV-HA [control rVV expressing influenza virus hemagglutinin (21)] and then analyzed via cytofluorography. Cell surface K^d expression was monitored by indirect immunofluorescence using the K^d-specific mAb SF1.1.1.

As seen in Fig. 3*A*, the fluorescent K^d-binding peptides bound to VV-K^d-infected cells at roughly twice the levels of background binding to VV-HA-infected cells. Specific binding to VV-$EC15K^d$-infected cells occurred at four times the level of binding to VV-K^d-infected cells. Only trace amounts of $EC15K^d$ molecules were expressed on cells (Fig. 3*B*), so the binding of fluorescent peptides was indicative of binding to intracellular molecules.

To investigate the binding of unconjugated peptides to intracellular K^d molecules, we performed competition assays. Coincubation of cells with K^d binding peptides reduced the binding of fluorescent peptides by 90%. The specificity of inhibition was demonstrated by the failure of K^b and K^k binding peptides to reduce the binding of fluorescent peptide binding. Thus, as with K^b, trafficking of peptides to intracellular K^d molecules in the ER did not require the presence of fluorescein. Important to note, the delivery of K^d binding peptides to the ER of VV-$EC15K^d$-infected cells in the absence of significant levels of cell surface K^d demonstrates that peptides are not transported to the ER after their capture by class I molecules expressed at the cell surface or, by inference, in an endosomal compartment.

Biochemical Evidence for Exogenous Peptide Binding to Newly Synthesized Class I Molecules. To obtain biochemical evidence for the delivery of exogenous peptides to the ER, RMA cells were preincubated with K^bFL for 30 min at 37°C, radiolabeled with [^{35}S]-methionine for 5 min, incubated on ice or at 37°C for 15 min, and then detergent-extracted. All radiolabeled K^b molecules are present in the ER of RMA cells after such brief labeling (8, 22). To recover class I molecules containing K^bFL from detergent extracts, we used protein A agarose loaded with anti-fluorescein antibodies. Postlysis association of K^bFL with K^b was minimized by inclusion of excess unconjugated peptide in the lysis buffer. Recovered material was analyzed by SDS/PAGE and visualized using a Phosphorimager. Proteins of several different mobilities were evident in the gel (Fig. 4). All but one were unrelated to binding of K^bFL to cellular proteins; they were recovered in similar amounts from cells not exposed to peptide. The single band uniquely present in K^bFL-exposed cells (Fig. 4, arrow) comigrates with K^b α chains recovered using anti-K^b antibodies. To determine to what extent peptide binding may have occurred subsequent to lysis under these conditions, [^{35}S]-methionine-labeled cells unexposed to K^bFL were coextracted with unlabeled, peptide-exposed cells ("mix"). Little (pulse) or no (chase) radiolabeled K^b was recovered, so we concluded that K^bFL binding to K^b occurred before detergent lysis and therefore represents binding to newly synthesized molecules in the ER.

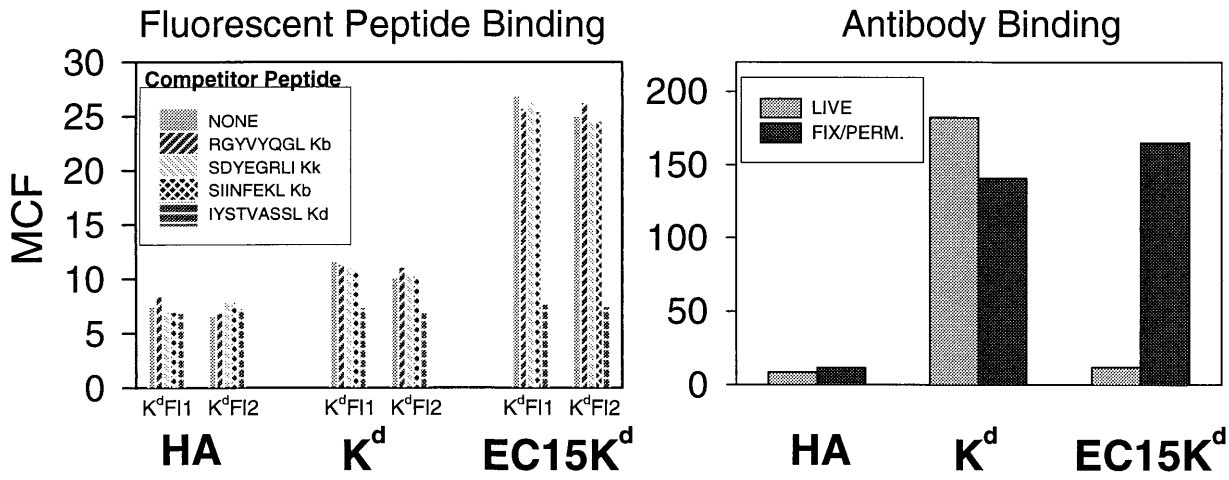

FIG. 3. Cytofluorographic analysis of K^dFL binding to VV-infected L cells. The binding of K^dFL1 and K^dFL2 to L cells infected with rVVs expressing K^d, ER-retained K^d, or influenza virus hemagglutinin in the presence or absence of the indicated competitor peptides was determined by cytofluorography. The expression of K^d by rVV-infected cells also was monitored by indirect immunofluorescence using the anti-K^d mAb SF1.1.1. (42). Cell surface expression was determined using live cells. To enable detection of intracellular K^d, cells were fixed and permeabilized. This results in the loss of surface K^d, accounting for the decrease in mAb binding to VV-K^d-infected cells (13).

Similar findings were made using LK^b cells, with the exception that, unlike RMA cells, in which a maximal signal was obtained immediately after pulse labeling, the signal increased after a 15-min chase period (Fig. 5*B*). Although other interpretations are possible, we believe that this indicates that peptide receptive molecules are formed more rapidly in RMA cells than in LK^b cells. In this experiment, we used BFA to explore whether peptide delivery to the ER entailed retrograde transport from the trans-Golgi complex, as has been reported for several toxins and other proteins (23–25). BFA prevents the retrograde delivery of endocytosed proteins to the ER by disconnecting the proximal and distal portions of the Golgi complex (26, 27). The activity of BFA in this experiment is confirmed by the slightly increased mobility of K^b α chains observed in the after-the-chase period, which reflects the trimming of N-linked oligosaccharides by enzymes in the ER and cis-Golgi complex (28, 29). Rather than inhibit the transport of K^bFL to the ER, BFA enhanced the recovery of K^b molecules containing the exogenously added peptide. This confirms the BFA-enhanced staining of 25-D1–16 on cells incubated with SIINFEKL and provides strong evidence that class I binding peptides are not delivered to the ER by retrograde transport through the secretory pathway. As with RMA cells, a cell mixing experiment (Fig. 5*A*) demonstrated that peptide binding occurs before cell lysis.

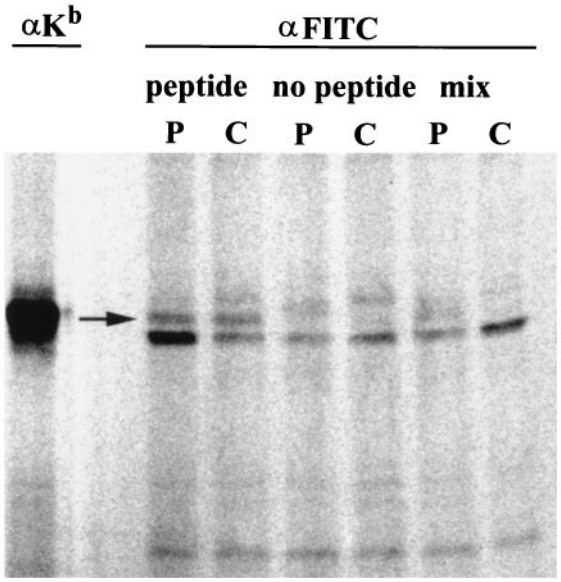

FIG. 4. Biochemical characterization of K^bFL binding to radiolabeled class I molecules. RMA cells incubated with or without K^bFL were pulse-radiolabeled with [^{35}S]-methionine and immediately incubated at 0°C (P) or chased for 15 min at 37°C (C). Detergent-soluble material bound to anti-fluorescein antibodies was analyzed by SDS/PAGE. In the last two lanes on the *Right*, peptide-exposed, unlabeled cells were mixed with radiolabeled cells not exposed to peptides before detergent extraction. Molecules comigrating with K^b recovered with specific antibodies are shown by the headed arrow.

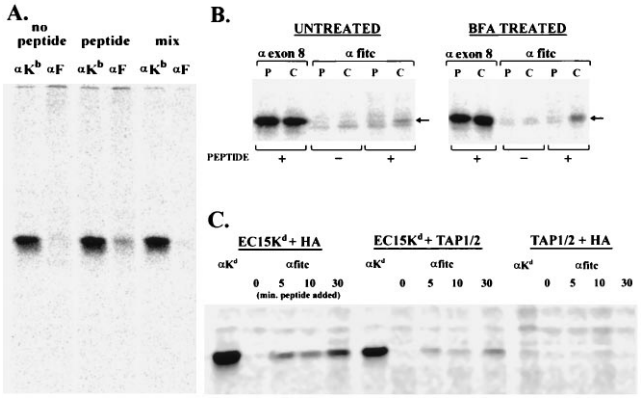

FIG. 5. Biochemical characterization of K^bFL binding to radiolabeled class I molecules: (A) LK^b cells were incubated with or without K^bFL for 30 min, labeled for 5 min with [^{35}S]-methionine, and chased for 15 min with unlabeled methionine. Mixing of cells was as in Fig. 4. Radiolabeled material recovered with K^b exon 8 or fluorescein (F)-specific antibodies was analyzed by SDS/PAGE. (B) LK^b cells untreated or pretreated for 2 h with 1 μg/ml BFA were incubated with or without K^bFL for 30 min labeled with [^{35}S]-methionine for 5 min and immediately incubated at 0°C (P) or chased for 15 min at 37°C (C). Radiolabeled material recovered with anti-K^b exon 8 or fluorescein-specific antibodies was analyzed by SDS/PAGE. Arrow locates K^b α chains specifically bound to anti-fluorescein antibodies. (*C*) T2 cells infected for 14 h with the rVV indicated were radiolabeled with [^{35}S]-methionine for 90 min. Cells were then incubated at 0°C (0) or at 37°C with 10 μg/ml K^dFL1 for 5, 10, or 30 min, and detergent-soluble material bound to anti-fluorescein antibodies was analyzed by SDS/PAGE. The lane marked anti-K^d represents K^d molecules collected from detergent extracts from cells not exposed to peptides using protein A agarose bound to the anti-K^d mAb SF1.1.1. Lysis buffer contained 5 μg/ml K^d-binding peptides IYATVAGSL and TYQRTRALV each.

In the next experiment, we examined the kinetics of peptide delivery to the ER in the presence and absence of TAP. T2 cells (a TAP-deficient human lymphoblastoid cell line) (12) were coinfected with VV-EC15K^d and either an rVV (VV-TAP[1&2]) coexpressing TAP1 and TAP2 (30) or an rVV (VV-HA) expressing influenza hemagglutinin as a control protein. Taking advantage of the retention of EC15K^d in the ER, cells were labeled with [^{35}S]-methionine for 90 min and then exposed to the K^{d}FL1 peptide for 5, 10, or 30 min at 37°C. [^{35}S]-methionine-labeled, detergent-soluble proteins reactive with anti-fluorescein antibodies were collected and analyzed by SDS/PAGE. An anti-fluorescein-reactive protein comigrating with EC15K^d was obtained from cells infected with VV-EC15K^d (Fig. 4*C*). The absence of this protein from cells coinfected with VV-HA and VV-TAP[1&2] confirms its identity as EC15K^d. Peptide delivery to EC15K^d molecules occurred within 5 min and doubled between 10 and 30 min. Coexpression of TAP did not enhance the kinetics of peptide delivery and indeed served only to decrease the amount of K^d recovered, an effect we attribute to increased competition for peptide binding by TAP-transported peptides. The binding of K^{d}FL1 to EC15K^d occurred before lysis of cells; in the same experiment, radiolabeled EC15K^d was not recovered with anti-fluorescein antibodies when unlabeled peptide-exposed cells were mixed with radiolabeled nonexposed cells before detergent extraction (not shown).

Based on this experiment, we conclude that TAP is not involved in the delivery of exogenous peptides to the ER and therefore that peptides are not delivered to the ER via the cytosol. Two additional findings support this conclusion. First, by laser scanning confocal microscopy, we found no significant quantitative or kinetic difference in the levels of K^{b}FL delivered to the ER of viable RMA and RMA/S cells. Second, direct delivery of K^{b}FL to the cytosol of LK^b cells via microinjection resulted in the accumulation of peptide in the nucleus with no apparent transport into the ER (not shown). Although we are uncertain as to the reason for nuclear accumulation of the peptide, our failure to detect the peptide in the nucleus of cells exposed to exogenous peptide provides an independent line of evidence that exogenous peptides are delivered to the ER directly in a membrane-bound compartment and do not transit the cytosol.

Peptide Delivery to the ER Is Inhibited in Parallel with Endocytosis. Given that the peptide does not traverse the cytosol, these findings implicate peptide delivery to the ER via a vesicular route. Vesicular trafficking of extracellular substances can be blocked by incubating cells either with azide in combination with 2-deoxyglucose to deplete cellular ATP levels or with aluminum fluoride, which is thought to act by inhibiting heterotrimeric G proteins (31). Addition of aluminum fluoride to LK^b cells after pulsing with K^{b}FL prevented ER staining and restricted the peptide to brightly staining vesicles in the cell periphery. These vesicles remained intensely fluorescent 3 h after exposure to peptide in aluminum fluoride-treated cells whereas, in untreated cells, vesicular fluorescence decreased approximately 5-fold (Fig. 1*C*). If aluminum fluoride was added to cells at the time of peptide addition, entry of peptide into vesicles was greatly diminished, and again, peptide was not delivered to the ER. Similar results were obtained using azide in conjunction with 2-deoxyglucose to deplete cellular ATP levels. The process is microtubule-independent, as nocodazole did not affect delivery of the peptide into the ER. We also examined the effects of temperature on peptide trafficking. Again, ER localization paralleled vesicular accumulation, with a partial effect observed at 26°C and near complete inhibition of both endocytosis and ER localization at temperatures of 20°C and below (not shown).

These findings provide additional evidence that peptides are not simply diffusing across membranes to access the ER. Rather, they strongly suggest that the peptide delivery to the ER occurs via a vesicular process that is inhibited in parallel with endocytosis.

DISCUSSION

We have demonstrated that MHC class I binding peptides are delivered from extracellular fluids to the ER where they bind class I molecules. Surface expression of an appropriate class I molecule is not required for this process, so peptide delivery does not represent the retrograde transport of class I molecules from the cell surface to the ER. The failure of unlabeled peptides specific for other class I molecules to inhibit delivery of K^{d}FL1 or K^{d}FL2 (Fig. 3) eliminates the possibility that another saturable receptor with broad specificity for peptides is involved in the process. This leads to the conclusion that peptides are delivered in fluid phase in a nonspecific manner.

Consistent with this conclusion, we detected peptide delivery to the ER of cells lacking a corresponding class I molecule. Peptide was present in the ER at low levels and rapidly disappeared. We believe that this indicates that peptides are delivered to the ER in a nonspecific manner and are quickly removed in the absence of a high affinity ligand. The loss of peptide from the ER of cells lacking the appropriate class I molecule is not blocked by BFA, so this cannot be exclusively caused by export through the secretory pathway. Indeed, given that the Golgi complex has numerous peptide-receptive molecules (13), our failure to detect peptides in the Golgi complex strongly suggests that peptides do not traffic through the outer portions of the secretory pathway. Class I binding peptides have been reported to be rapidly exported from the ER to the cytosol in a or semi-intact system where they are rapidly destroyed by cytosolic proteases (32). Additionally, there may be resident ER proteases that contribute to peptide loss.

These conclusions predict that nonclass I binding peptides will be detected in the ER given the expression of ligand of sufficiently high affinity to prevent their export or destruction. In additional experiments, we could show that fluorescein-conjugated versions of synthetic peptides known to bind BiP (FWGLWPWEASGSAK-fluorescein) (33) are transported to the ER of viable cells from the culture media (unpublished results). Their staining of the ER was less intense than class I binding peptides, being only slightly above the threshold for detection above background fluorescence. This may reflect the relatively low affinity of peptides for BiP (10^5 vs. 10^7 to 10^8 M^{-1} for class I binding peptides).

There are several previous examples of delivery of extracellular material to the ER. A number of toxins are thought to be delivered to the ER via retrograde transport through the secretory pathway using a mechanism whose primary purpose is the retrieval of proteins meant to reside in the ER (26, 27). SV40 virions are transported from the extracellular media to the ER, but the route of transport is uncertain and may also use the ER retrieval mechanism (34). Acylated tripeptides added to cells were shown to acquire N-linked oligosaccharides, indicative of transport to the ER (35). The mechanism of transport was not examined, but glycosylation was thought to be dependent on the presence of the acyl group, and it was presumed that the hydrophobicity of the peptides allowed them to diffuse through the plasma and ER membranes. Evidence has been presented for the direct ER delivery of caveolin from the cell surface (36). Both RMA and T2 cells are of lymphocytic origin, and the existence of caveolae in lymphocytes is highly questionable (37). Moreover, we are unaware of evidence that extracellular material accompanies caveolin to the ER.

In the present study, we showed that extracellular peptide transport to the ER is unlikely to use the ER-retrieval mechanism because it is not blocked by BFA. The TAP-independence of the process, in conjunction with the discrepancy between K^{b}Fl localization in the nucleus of cells micro-

injected with the peptide vs. those simply incubated with the peptide, demonstrates that peptides are not entering the ER via the cytosol. Based on these considerations, we conclude that the delivery of peptides to the ER occurs via an intracellular trafficking pathway, present in a variety of cell types, that functions in the fluid phase communication between the extracellular medium and the ER.

Although the pathway is inhibited in parallel with pinocytosis, this does not necessarily indicate a role for pinosomes in the pathway. Pinosomes may be the source for the ER-targeted vesicles, but equally (or perhaps even more) plausible is that vesicles originate at the plasma membrane and have the same general requirements in their formation as pinocytic vesicles. The pathway may be limited to small molecules (the peptides we used have a M_r of <2000) since prolonged (>12 h) incubation of cells with β_2-microglobulin (M_r 12000) does not result in detection of β_2-microglobulin in the ER (unpublished observations) and does not rescue the ability of β_2-microglobulin-deficient cells to present endogenous antigens to T cells (38).

These findings are directly relevant to interpretation of the numerous instances in which class I binding peptides are used *in vivo* to stimulate immune responses or for *in vitro* sensitization of target cells. It is clear that exogenous peptides can bind to class I molecules present at the cell surface. This follows from the ability of exogenous β_2-microglobulin to enhance the binding of synthetic peptides (38, 39) and peptide binding to cells at low temperatures (13). At temperatures >20°C, however, the present findings demonstrate that peptide binding will also occur in the ER. This would account for the findings of Rock *et al.* (40) that β_2-microglobulin-independent target cell sensitization with exogenous peptides is energy-dependent. Peptide trimming in the ER can contribute to the formation of class I binding peptides (14, 41), so the antigenicity and immunogenicity of extended exogenous peptides may be enhanced after their transport to the ER.

The biological significance of these findings is not limited to antigen presentation. Indeed, the primary function of the pathway may be nonimmunological in nature. The vesicular delivery of small molecules to the ER has obvious implications for cell biology. First, the pathway may contribute to maintaining the characteristic ER solute composition. Second, given the apparent vesicular nature of the pathway, it may play a role in intracellular lipid economy. Third, the pathway may act in signal transduction. Many peptide hormones are of similar size to class I binding peptides and should also be transported to the ER along with small charged organic molecules active in cell signaling. Localization of receptors to the ER would provide distinct advantages over cell surface receptors. Receptors located in inner portion of the nuclear membrane would be able to transmit signals directly to the nucleus, entirely bypassing the requirement for nuclear localization of cytosolic targets. Cell surface receptors begin their existence in the ER, so it is possible that some are active shortly after their synthesis. In this circumstance, the signals delivered by ER and cell surface receptors may differ qualitatively, providing a rationale for dual location. It is also possible that some receptors reside exclusively in the ER and depend entirely on delivery of their ligands to the ER.

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