The B-subunit component of *Escherichia coli* heat-labile enterotoxin (EtxB), which binds to cell surface GM1 ganglioside receptors, was recently shown to be a highly effective vehicle for delivery of conjugated peptides into the major histocompatibility complex (MHC) class I pathway. In this study we have investigated the pathway of epitope delivery. The peptides used contained the epitope either located at the C terminus or with a C-terminal extension. Pretreatment of cells with cholesterol-disrupting agents blocked transport of EtxB conjugates to the Golgi/endoplasmic reticulum, but did not affect EtxB-mediated MHC class I presentation. Under these conditions, EtxB conjugates entered EEA1-positive early endosomes where peptides were cleaved and translocated into the cytosol. Endosome acidification was required for epitope presentation. Purified 20 S immunoproteasomes were able to generate the epitope from peptides in vitro, but 26 S proteasomes were not. Only presentation from the C-terminal extended peptide was proteasome-dependent in cells, and this was found to be significantly slower than presentation from peptides with the epitope at the C terminus. These results implicate the proteasome in the generation of the correct C terminus of the epitope and are consistent with proteasome-independent N-terminal trimming. Epitope presentation was blocked in a TAP-deficient cell line, providing further evidence that conjugated peptides enter the cytosol as well as demonstrating a requirement for the peptide transporter. Our findings demonstrate the utility of EtxB-mediated peptide delivery for rapid and efficient loading of MHC class I epitopes in different cell types. Conjugated peptides are released from early endosomes into the cytosol where they gain access to proteasomes and TAP in the “classical” pathway of class I presentation.

Cytotoxic CD8+ T lymphocytes recognize and clear host cells infected with intracellular bacteria or viruses, or tumors that display non-self or tumor-derived peptides on their surface in the context of major histocompatibility complex (MHC) class I molecules (1). The majority of peptides presented are generated from endogenously synthesized proteins that are degraded by the proteasome (reviewed in Refs. 2 and 3). These are subsequently transported into the lumen of the endoplasmic reticulum (ER) by the transporter associated with antigen presentation (TAP). In the ER, peptides bind to newly synthesized MHC class I molecules, which are then transported to the cell surface for recognition by CD8+ T-cells. This leads to T-cell activation, and eventually to cytotoxic CD8+ T lymphocyte-mediated lysis of peptide-presenting cells. Activation of cytotoxic CD8+ T lymphocytes is obviously an important strategy for vaccination. However, exogenous soluble antigens generally fail to gain access to the cytosolic compartment of cells, and thus novel strategies of targeting this pathway are needed.

One strategy that is receiving attention is the use of protein delivery vehicles that possess an inherent capacity to enter mammalian cells (reviewed in Refs. 4 and 5). This is exemplified by bacterial A-B type toxins that, following binding by the B component to cell surface receptors, deliver the A component into the cytosol. Several investigators have attempted to exploit such toxins by fusing antigens to the toxin A component. For example, inactive derivatives of diphtheria toxin (6), adenylyl cyclase toxin of *Bordetella pertussis* (7), and anthrax toxin (8) have been successfully used to deliver fused or conjugated epitopes into the MHC class I pathway. Recently, we reported that the B-subunit of *Escherichia coli* heat-labile enterotoxin (EtxB) can be used as a delivery vehicle for model class I epitopes, namely the SIINFEKL epitope of chicken ovalbumin and the ASNENMETM nucleoprotein epitope from influenza virus (9). In these instances, epitope presentation by MHC class I molecules was found to require conjugation of peptides to EtxB and binding of the EtxB-peptide conjugates to GM1 ganglioside receptors. Moreover, a significant enhancement in the efficiency of epitope presentation was achieved by incorporating, adjacent to the epitope, a sequence derived from the DNA polymerase (Pol) of herpes simplex virus type 1. This 10-amino acid stretch of non-polar and charged residues (AV-GAGATAEE), termed the Pol-loop segment, has been proposed...
to have an intrinsic propensity to insert into lipid bilayers, thereby enhancing translocation (10).

When an EtxB conjugate containing a 31-mer peptide (CEKLAGFGAVGATAGAESINEFKLEWTS) with an internal SIINFEKL epitope was evaluated, epitope presentation on MHC class I molecules was found to be inhibited by addition of epoxomicin, an inhibitor of the proteasome. This finding is consistent with epitope entry into the cytosol. However, because proteasomes are involved in ER-associated protein degradation as well as proteolysis of cytosolic proteins and several other toxins are known to exploit the retrograde transport system, the route of peptide delivery was not clear. Confocal microscopy of the uptake of EtxB-peptide conjugates showed the EtxB moiety in the Golgi/ER compartment. Because presentation of the SIINFEKL epitope by the EtxB-19-mer conjugate is so rapid, the possibility remains that the epitope may be delivered directly into the Golgi/ER where loading of MHC class I molecules could occur.

In this study we have investigated the trafficking pathway of EtxB-peptide conjugates to assess whether epitope presentation is because of peptide translocation into the cytosol or direct EtxB-mediated peptide delivery into the ER. The results demonstrate (i) that EtxB-mediated epitope presentation does not require trafficking of the EtxB moiety to the Golgi/ER, (ii) that epitope translocation into the cytosol occurs from an acidified endocytic compartment, (iii) that immunoproteasomes can generate the active epitope in vitro but presentation is only proteasome-dependent in cells when a C-terminal extended epitope is used, (iv) that this pathway is not specific to dendritic cells, and (v) that presentation requires a functional TAP transporter.

**EXPERIMENTAL PROCEDURES**

**Production of EtxB Conjugates**—Recombinant EtxB was expressed in a nonoxigenic marine vibrio, *Vibrio* sp. 60, and purified as previously described (11). Purified preparations of EtxB were depleted of lipopolysaccharide using Detoxi-Gel™ Affinity Pak™ columns (Pierce) and contained <50 endothelin units/mg of protein as determined in a Limulus amoebocyte lysate assay (BioWhittaker, Walkersville, MD). The epitope sequence in this study was synthesized by solid-phase synthesis using reverse-phase high-performance liquid chromatography, and their molecular masses confirmed by mass spectrometry. The peptides were used to detect the level of the SIINFEKL epitope bound to MHC class I molecules at the surface of the antigen presenting cells (14). On recognition of the epitope these cells produce IL-2. Therefore epitope presentation by JAWSII dendritic cells, E36.12.4 hamster lung carcinoma cells, and RMA lymphoma cells were examined by monitoring the IL-2 released. JAWSII cells were seeded in 96-well plates at 5 × 10^4 cells/well and cultured overnight. E36 cells were seeded at 1–5 × 10^4 for 2–4 h, and RMA and RMA-S cells were seeded at 1 × 10^6/ml for 2 h prior to the addition of test samples. Duplicate aliquots of cells were incubated for 2 h with duplicates of each test sample that contained peptide alone, peptide admixed with EtxB, or EtxB-peptide conjugates, all at a concentration of 100 nm peptide. Treatment of cells with the peptide (to give the maximal level of SIINFEKL bound to MHC I) and PBS alone were used as positive and negative controls, respectively. Following incubation with antigen, cells were fixed for 10 min with 1% (v/v) paraformaldehyde in phosphate-buffered saline (PBS) at room temperature, washed five times with medium, and incubated overnight with RF33.70 T-cell hybridoma seeded at 5 × 10^5 cells/well. Following overnight incubation the T-cell hybridoma was detected using a commercially available IL-2 enzyme-linked immunosorbent assay kit, which was used according to the instructions of the manufacturer (BD Pharmingen). IL-2 levels are given as mean units per ml ± S.E. Presented data are representative of at least three independent experiments. Statistical significance was assessed by the Student’s t test. A probability value (p) of <0.05 was regarded as statistically significant.

The inhibitory effects of filipin (10 μg/ml), methyl-β-cyclodextrin (MCD) (10 mM), bafilomycin A1 (BafA1), brefeldin A, leupeptin (100 μM), pepstatin (100 μM), bestatin (100 μM) (all from Sigma), and epoxomicin (10 μM), lactacytin (10 μM), and MG-132 (10 μM) (all from Calbiochem, Nottingham, UK) on EtxB-mediated epitope delivery were also studied. In these experiments JAWSII cells were pretreated with inhibitors 1 h prior to the addition of the EtxB-conjugated IL-2 peptide. The presence of the inhibitor cells was present throughout the duration of the incubation of the cells with antigen. When cells were incubated with filipin and an inhibitor, the filipin inhibitor cells were first incubated with filipin for 1 h and then incubated with filipin plus the second inhibitor for 1 h.

**Degradation of Peptides by Purified Proteasomes**—Rat liver 20 S and 28 S proteasomes and rat spleen 20 S immunoproteasomes, which contain the β1i- and β1i-subunits, were used (kindly provided by K. Forti, Department of Biochemistry, University of Bristol) and purified as previously described (15, 16). The ability of purified proteasome to generate mature MHC class I epitope was investigated as follows. Peptide at a final concentration of 12 pmol/ml in PBS was incubated with proteasome (2 μg) for 6 or 8 h at 37 °C and then incubated for 5 min with JAWSII cells at a final concentration equivalent to 350 nM peptide. In all experiments the proteasome was preincubated for 1 h with epoxomicin (10 μM) before addition of the peptide. The cells were then fixed with 1% (v/v) paraformaldehyde and antigen presentation was analyzed as above.

**Confocal Microscopy**—For analysis by immunofluorescence microscopy, JAWSII cells were first grown for 48 h on sterile coverslips coated with poly-L-lysine (Sigma). The cells were then washed with PBS and incubated with either antibodies against early endosomal antigen 1 (EEA1) (Santa Cruz Biotechnology, Santa Cruz, CA) or lysosome-associated membrane protein 2 (Affinity BioReagents, Golden, CO), specific markers for the early endosomes (17) and lysosomes (18), respectively, or rhodamine-labeled wheat germ agglutinin (WGA), to visualize plasma and Golgi membranes, all diluted in PBS/bovine serum albumin (PBS/bovine serum albumin (PBS containing 3% bovine serum albumin, fraction V, Sigma). The cells were then washed with PBS and incubated for 1 h with either antibodies against early endosomal antigen 1 (EEA1) (Santa Cruz Biotechnology, Santa Cruz, CA) or lysosome-associated membrane protein 2 (Affinity BioReagents, Golden, CO), specific markers for the early endosomes (17) and lysosomes (18), respectively, or rhodamine-labeled wheat germ agglutinin (WGA), to visualize plasma and Golgi membranes, all diluted in PBS/bovine serum albumin. Next, coverglasses were washed three times with PBS and incubated with fluorescein isothiocyanate- or TRITC-labeled secondary antibodies directed against either mouse or rabbit immunoglobulin G (Jackson ImmunoResearch Laboratories) in PBS/bovine serum albumin for 1 h. The washed coverglasses were then mounted and fixed onto glass examination slides spotted with Mowiol containing 2.5% 1,4-diazabicyclo[2.2.2]octane (DABCO) anti-fading and the DNA-binding agent 4',6-diamidino-2-phenylindole dihydrochloride (1 mg/ml) for nuclear staining. Coverglasses were mounted and fixed onto glass examination slides spotted with Mowiol containing 2.5% 1,4-diazabicyclo[2.2.2]octane (DABCO) anti-fading and the DNA-binding agent 4',6-diamidino-2-phenylindole dihydrochloride (1 mg/ml) for nuclear staining.
Filipin Inhibits Trafficking of EtxB Peptide Conjugates—The ubiquitously expressed glycosphingolipid ganglioside GM1, which is highly enriched in lipid rafts, is the main functional receptor of Etx and its close homologue Ctx (19). Depletion, redistribution, or removal of plasma membrane cholesterol by the sterol-binding agents, filipin and MCD, disrupt lipid rafts and have been shown to inhibit trafficking of the toxins to the Golgi/ER (22). In an attempt to determine whether the trafficking of EtxB-peptide conjugates in JAWSII dendritic cells is inhibited by filipin and MCD and whether or not this affects efficient EtxB-mediated epitope delivery, the cells were treated with 10 μg/ml filipin or 10 mM MCD for 1 h prior to addition of the EtxB-19-mer or EtxB-31-mer peptide conjugates containing the ovalbumin-derived SIINFEKL epitope. Confocal microscopy revealed that filipin inhibited the intracellular trafficking of the EtxB-peptide conjugates (Fig. 1). Monoclonal antibody 118-8, specific for the EtxB pentamer was used to monitor the trafficking of the EtxB moiety, whereas rhodamine-labeled WGA, which has affinity for N-acetylated β-N-glucosaminyl residues and N-acetylated β-N-glucosamine oligomers, was used to stain plasma and Golgi/ER membranes (22). In the absence of filipin, it can be seen that 2 h after addition of either the EtxB-19-mer or EtxB-31-mer conjugates, the EtxB moieties are clearly detected at the cell surface and in the perinuclear region, indicating toxin binding and internalization (Fig. 1A, images a–d). The co-localization of the internalized EtxB with WGA is indicative of conjugate trafficking to the Golgi/ER (Fig. 1A, images c and d). By contrast, when cells were pretreated with filipin, the EtxB moieties of the conjugates were found in a location proximal to the cell surface, with none in a perinuclear location (Fig. 1B, images a–d). The detected EtxB moieties did not appear to be in the same location as surface-localized WGA, as the fluorophors were not entirely superimposable (compare Fig. 1B, image c, with Fig. 1A, image c); or to be present in lysosome-associated membrane protein 2 containing lysosomes (Fig. 1B, images e–h). Strikingly, the EtxB moieties present in the filipin-treated cells fully co-localized with the early endosomal marker, EEA1 (Fig. 1B, images i–l). Identical results were also obtained when the cells were treated with MCD prior to EtxB conjugate uptake (data not shown). We therefore conclude that disruption of cholesterol-containing membrane microdomains by filipin or MCD blocks EtxB conjugate trafficking to the Golgi, but permits uptake into an early endosomal EEA1-positive compartment.

Filipin and MCD Do Not Inhibit Antigen Presentation—To determine whether the failure of the EtxB-peptide conjugates to traffic to the Golgi/ER affects EtxB-mediated epitope delivery, JAWSII cells were treated with 10 μg/ml filipin or 10 mM MCD prior to addition of the EtxB-19-mer or EtxB-31-mer peptide conjugates and then the presentation of the SIINFEKL epitope was monitored in a standard antigen presentation assay by measuring IL-2 release by the SIINFEKL-specific RF33.70 T-cell hybridoma. In these experiments, free 8-mer SIINFEKL peptide was used as a positive control. This peptide can displace peptides already bound to MHC class I molecules on the cell surface, without the need for intracellular processing. We found that treatment with filipin had no significant effect on SIINFEKL epitope presentation using the EtxB-19-mer or EtxB-31-mer conjugates (Fig. 2A). Similar levels of SIINFEKL epitope presentation were also observed when using the free 8-mer SIINFEKL epitope at an equivalent peptide concentration, and importantly, treatment of JAWSII cells with filipin did not affect the direct loading of the free 8-mer peptide. Results obtained with MCD were in complete agreement with the data obtained with filipin (Fig. 2B). Accordingly, the extent of epitope presentation observed following treatment with MCD and the EtxB-19-mer and EtxB-31-mer conjugates were comparable with those obtained in the absence of MCD or with free 8-mer SIINFEKL peptide. MCD also had no affect on the direct loading of the free 8-mer peptide. Taken together, these findings demonstrate that trafficking of EtxB conjugates to an early endosomal compartment is sufficient for EtxB-mediated epitope delivery into the MHC class I antigen presentation pathway.

Epi-tope Presentation in Filipin-treated Cells Requires Endosomal Acidification—BafA1, an inhibitor of endosomal acidification (23), prevents EtxB-mediated epitope presentation (9). JAWSII cells were pretreated with 10 μg/ml filipin and 200 nM BafA1 as described under “Experimental Procedures” prior to the addition of the EtxB-19-mer or EtxB-31-mer conjugates. As shown in Fig. 3, addition of BafA1 to filipin-treated JAWSII cells strongly inhibited the ability of EtxB to deliver the SIINFEKL epitope into the MHC class I presentation pathway (compare Fig. 3, A and B). This implies that the early endosomal compartments in which EtxB conjugates accumulate in filipin-treated cells (Fig. 1B, image i–l), must be acidified for efficient peptide release and antigen presentation.

![Image](https://via.placeholder.com/150)
In filipin-treated cells brefeldin A, which disrupts the Golgi complex (24), prevented epitope delivery from both the EtxB-19-mer and EtxB-31-mer conjugates (Fig. 3, C and D). These results demonstrate that even in the presence of filipin an intact Golgi is required, presumably to allow trafficking of peptide-MHC class I complexes to the cell surface. Epoxomicin, a potent proteasome inhibitor (25), was found to selectively inhibit epitope delivery from the EtxB-31-mer but not the EtxB-19-mer conjugate whether cells were treated with filipin or not (Fig. 3, E and F).

**Effects of Protease Inhibitors on EtxB-mediated Epitope Delivery** —To further characterize epitope generation during EtxB-mediated epitope delivery of conjugated peptides into the MHC class I pathway, the effects of a range of protease inhibitors were investigated. These included: (i) leupeptin, an inhibitor of lysosomal serine and cysteine proteases (26); (ii) pepstatin, an inhibitor of aspartic proteases, cathepsin D (27), and cathepsin E (28); (iii) bestatin, an aminopeptidase inhibitor (29); and (iv) protease inhibitors epoxomicin (25), lactacystin (30), and MG-132 (31). JAWSII cells were preincubated with inhibitor for 1 h, after which 100 nM EtxB-19-mer or -31-mer conjugate was added. Table I shows that treatment with leupeptin, pepstatin, and bestatin had no effect on the ability of the conjugates to deliver the SIINFEKL epitope into the MHC class I presentation pathway in JAWSII cells. Lactacystin and MG-132 inhibited antigen presentation when the cells were treated with the EtxB-31-mer conjugate. In contrast, epitope presentation from the EtxB-19-mer peptide conjugate, in which the SIINFEKL epitope is located at the C terminus of the peptide was not inhibited by addition of proteasome inhibitors. The results are consistent with the proteasome generating the correct C terminus of the SIINFEKL epitope following peptide translocation into the cytoplasm.

**Proteasome-dependent Generation of the SIINFEKL Epitope by Purified Proteasomes** —Given that it is virtually impossible to detect free peptide in the cytoplasm (32) and assuming that the peptides are translocated into this compartment, where they would come into contact with the proteasome, we next investigated whether 20 S proteasomes (immunoproteasomes) could generate the SIINFEKL epitope from our 19- and 31-mer peptides in vitro. Fig. 4A shows that when the peptides were incubated with purified immunoproteasomes in vitro, and the degradation products subsequently added to JAWSII cells, epitope presentation could readily be detected. In addition, generation of the SIINFEKL epitope from the 19-mer and 31-mer peptides was completely inhibited by preincubation of immunoproteasome with epoxomicin. In contrast, when the peptides alone were incubated with JAWSII cells, no SIINFEKL epitope presentation was evident. This demonstrates that SIINFEKL peptides were being generated by the purified immunoproteasome and were capable of competing for bound peptides present on already displayed MHC class I complexes such as the 8-mer peptide. Immunoproteasomes purified from spleen were used for these experiments because dendritic cells are known to have a high content of immunoproteasomes (33). Essentially identical results were obtained with 20 S proteasomes purified from rat liver. These findings indicate that the 20 S proteasome is proficient in generating the SIINFEKL epitope from both the 31-mer and 19-mer peptides. However, purified 26 S proteasomes were unable to produce the SIINFEKL epitope when tested at the same protein concentration (Fig. 4B). Because 26 S proteasomes have a higher activity than 20 S proteasomes in standard assays (16) this result suggests that the 31-mer peptide does not gain access to the 26 S proteasome catalytic sites.

The EtxB-31-mer Conjugate Displays a Marked Alteration in the Kinetics of Epitope Delivery—The kinetics of appearance of MHC class I-SIINFEKL complexes on the cell surface following treatment of cells with EtxB-19-mer, -26-mer, and -31-mer conjugates was evaluated by fixing the cells at various time points after incubation with the conjugates. After 5 min of incubation with the conjugates, no peptide presentation was evident (Fig. 5). However, after 15 min incubation with the EtxB-19-mer and -26-mer peptide conjugates, maximal presentation levels were attained with epitope delivery showing similar kinetics. In contrast, the 31-mer conjugate displayed markedly different kinetics of epitope delivery. For example, after 20 min incubation with the EtxB-31-mer conjugate no epitope presentation was evident, whereas maximal levels of presentation, comparable with that of the EtxB-19-mer and EtxB-26-mer conjugates, was reached after 60 min incubation. Thus the extension of the conjugated peptide on the C terminus of the epitope appears to alter the kinetics of delivery of peptides into the MHC class I pathway. The increase in time taken for presentation is consistent with an additional rate-limiting step (possibly cleavage by the proteasome) being required for epitope liberation from the 31-mer peptide.

**Demonstration of TAP-dependent Presentation** —The TAP transporter is normally involved in the translocation of epitopes from the cytoplasm into the lumen of the ER. Once in the ER, peptides bind to and stabilize newly synthesized MHC class I molecules. Investigation of the role of TAP in EtxB-mediated epitope delivery would therefore allow us not only to determine whether this transporter was required but also to provide further evidence that the conjugated peptides do indeed enter the cytoplasm. The role of TAP in EtxB-mediated epitope delivery was investigated in antigen presenting assays using a TAP-deficient cell line, RMA-S with its parent cell line, RMA, as a control. Fig. 6 demonstrates that when RMA cells were treated with EtxB-19-mer or EtxB-31-mer conjugates the levels of epitope presentation were similar to those observed with the free 8-mer SIINFEKL peptide. In contrast, substantially (>75%) reduced amounts of epitope presentation were observed when the EtxB-19-mer and EtxB-31-mer conjugates.
of the shorter peptide. Because BafA1 was found to inhibit cleavage of the peptide from the conjugate or reduced stability of the shorter peptide, we conclude that both the 19-mer and 31-mer peptides enter the cytoplasm and that epitope delivery from both peptide conjugates requires a functional TAP.

**Eptope Delivery in Different Cell Types**—Characterization of EtxB-mediated antigen presentation in E36.12.4 lung carcinoma cells showed broadly similar characteristics to that in the dendritic cell line. SIINFEKL was presented from 19-mer, 26-mer, and 31-mer conjugates but not from free peptides alone or in the parent cell line RMA. When RMA cells were pretreated with epoxomicin, to specifically inhibit the proteasome, presentation from the EtxB-19-mer conjugate was completely blocked. We therefore conclude that both the 19-mer and 31-mer peptides enter the cytoplasm and that epitope delivery from both peptide conjugates requires a functional TAP.

**Table I**

| Inhibitor     | EtxB-19-mer | EtxB-31-mer |
|---------------|-------------|-------------|
| None          | 353.9 ± 1.0 | 319.2 ± 4.2 |
| Leupeptin     | 338.0 ± 27.6| 329.6 ± 1.0 |
| Pepstatin     | 355.8 ± 1.0 | 318.2 ± 2.6 |
| Bestatin      | 357.5 ± 11.6| 321.8 ± 9.0 |
| Lactacystin   | 344.7 ± 17.1| 6.3 ± 2.4   |
| MG-132        | 357.0 ± 2.0 | 5.2 ± 1.9   |

were added to RMA-S cells. Importantly, when 8-mer peptide was used to load MHC class I molecules on the surface of RMA-S cells, similar levels of presentation were observed to that of the 8-mer in the parent cell line RMA. When RMA cells were pretreated with epoxomicin, to specifically inhibit the proteasome, presentation from the EtxB-19-mer conjugate was unaffected (Fig. 6B), whereas epitope delivery by the EtxB-31-mer conjugate was completely blocked. We therefore conclude that both the 19-mer and 31-mer peptides enter the cytoplasm and that epitope delivery from both peptide conjugates requires a functional TAP.

**Fig. 3.** Effects of inhibitors on EtxB-induced antigen presentation following incubation with filipin. Following preincubation of cells with filipin, the effects of inhibitors on EtxB-mediated delivery of the SIINFEKL epitope into the MHC class I processing and presentation pathway were determined. JAWSII cells were seeded in 96-well plates at 2 × 10^5/ml and cultured overnight. Cells were incubated for 1 h in the absence (panels A, C, and E) or presence (panels B, D, and F) of 10 μg/ml filipin before further incubation of controls or treatment with inhibitors. For the inhibitor treatments cells were incubated for 1 h with 200 nM BafA1 (A and B), 10 μM brefeldin A (C and D), or 10 μM epoxomicin (E and F). Epitope delivery from the EtxB-19-mer and EtxB-31-mer conjugates was then determined by incubation of treated cells with 100 nM conjugate for 2 h. The 8-mer SIINFEKL peptide and PBS alone were used as positive and negative controls, respectively. After subsequent fixing and washing of the JAWSII cells, they were incubated overnight with RF33.70 cells and then IL-2 production was assayed using an enzyme-linked immunosorbent assay.

**Fig. 4.** Generation of the SIINFEKL epitope from the 19-mer and 31-mer peptides by purified proteasomes in vitro. Generation of the SIINFEKL epitope from the 19-mer and 31-mer peptides by proteasomes was assessed by incubation of JAWSII cells with their proteasome-derived cleavage products. A, spleen immunoproteasomes were incubated in PBS for 6 h at 37 °C with 31-mer or 19-mer peptides (24 pmol/ml). Prior to the digest, the immunoproteasomes were pretreated for 1 h with or without epoxomicin (10 μM) to confirm proteasome-dependent generation of SIINFEKL. After snap freezing the digests were stored at −20 °C prior to assay of the SIINFEKL produced as follows. JAWSII cells were cultured overnight and then incubated for 5 min with samples of peptide digests equivalent to a final peptide concentration of 250 nM. Assays were carried out in duplicate with 8-mer SIINFEKL peptide and PBS alone as positive and negative controls, respectively. Cells were fixed, washed, and incubated overnight with the RF33.70 cells. The production of IL-2 was proportional to the amount of SIINFEKL present in the digests. A, comparison of generation of the SIINFEKL epitope by purified rat liver 20 S and 26 S proteasomes was assessed as described in A by incubation of JAWSII cells with the cleavage products of the 31-mer peptide.
DISCUSSION

Bacterial A-B type toxins are emerging as an important group of delivery vehicles for transporting peptides into the MHC class I pathway of mammalian cells (4, 5). To date, most studies have focused on inserting heterologous peptides into the part of the bacterial toxin that can enter the cytosol. For example, Olsnes and co-workers (6) engineered onto the N-terminus of diphtheria toxin a range of peptides, including class I epitopes from influenza virus matrix and nucleoprotein, and showed that the N-terminal A-fragment, containing the epitope, was translocated to the cytosol (6). Similarly, a 254-

amino acid N-terminal fragment of the lethal factor (an “A-component” of anthrax toxin) was shown to be highly effective in delivering fused epitopes, or even larger polypeptides, into the cytosol (8, 34). However, the precise route of presentation depends on the toxin and in many cases does not involve proteasomes and TAP. For example, inactivated pertussis toxin can deliver a 9-amino acid class I epitope from HIV gp120 without involvement of the cytosolic class I antigen processing pathway (35). In this instance, the use of lactacystin or a TAP-deficient cell line failed to prevent epitope presentation. Delivery with Shiga toxin is inhibited by lactacystin but the toxin enters the cell by multiple routes (36). It can follow the retrograde transport system directly to the ER or gain access to late endosomes/lysosomes.

The non-toxic B-subunit component of E. coli enterotoxin is an effective vehicle for delivery of conjugated epitopes into the MHC class I pathway (9) and the time course shows that the process is very rapid. EtxB binds to GM1 ganglioside receptors at the cell surface but does not insert into the membranes per se. It remains on the luminal surface of internalized endocytic vesicles that are trafficked to the Golgi/ER compartment. However, the results presented here further define the route of peptide delivery and demonstrate that this is not the case. Following entry of EtxB-peptide conjugates into early endosomes, the conjugated peptide is liberated and translocated directly from the endosomal compartments into the Golgi/ER compartment. This is evident from the kinetics of antigen presentation using EtxB-19-mer, -26-mer, and -31-mer conjugates. The time course of antigen presentation was measured by incubating JAWSII cells for various times (0, 5, 10, 15, 20, 30, 60, or 120 min) with 19-mer (CAVGAGATAEESIINFEKL), 26-mer (CEKLAGGFGAVGATAEESIINFEKL), and 31-mer (CEKLAGGFGAVGATAEESIINFEKLTEWTS) EtxB-peptide conjugates. The IL-2 produced during incubation of the antigen presenting JAWSII cells with the T cell line was assayed as a measure of antigen presentation.

FIG. 5. Kinetics of antigen presentation using EtxB-19-mer, -26-mer, and -31-mer conjugates. The time course of antigen presentation was measured by incubating JAWSII cells for various times (0, 5, 10, 15, 20, 30, 60, or 120 min) with 19-mer (CAVGAGATAEESIINFEKL), 26-mer (CEKLAGGFGAVGATAEESIINFEKL), and 31-mer (CEKLAGGFGAVGATAEESIINFEKLTEWTS) EtxB-peptide conjugates. The IL-2 produced during incubation of the antigen presenting JAWSII cells with the T cell line was assayed as a measure of antigen presentation.

FIG. 6. TAP dependence of antigen presentation from EtxB-19-mer and EtxB-31-mer conjugates. Epitope presentation by EtxB-19-mer and EtxB-31-mer was measured in the RMA and RMA-S (TAP-deficient) cell lines. Cells were preincubated for 1 h without (A) or with (B) epoxomicin (10 μM) to inhibit proteasome activity. Cells were then incubated with 100 nM EtxB-19-mer or EtxB-31-mer for 2 h. The 8-mer SIINFEKL peptide was used for a positive control to show the maximum level of presentation and PBS alone was the negative control. Following incubation with peptide or conjugate, cells were fixed, washed, and incubated overnight with RF33.70 T-cells prior to measurement of IL-2 production. Levels of IL-2 produced were significantly reduced in experiments using TAP-deficient RMA-S cells incubated with EtxB-peptide conjugates (compared with levels obtained with the 8-mer SIINFEKL peptide, *p < 0.05).

FIG. 7. Antigen presentation from EtxB conjugates in E36.12.4 cells. A, EtxB-mediated delivery of epitope was tested in the hamster lung carcinoma cell line, E36.12.4. Cells were incubated for 2 h at a final concentration of 10 nM peptide with, peptide alone, peptide admixed with EtxB, or EtxB-peptide conjugates, as indicated. Free 8-mer peptide was used as positive control. Antigen presentation was assayed by measuring IL-2 released after overnight incubation of E36.12.4 cells with RF33.70 T-cells. Duplicate samples were tested and similar results were obtained in at least two separate experiments. B, the effects of the inhibitors of endosomal acidification (BafA1, 200 nM) and proteasomes (epoxomicin, 10 μM) were tested on antigen presentation from EtxB-31-mer. E36.12.4 cells were treated with inhibitor for 1 h and then incubated with 100 nM EtxB-31-mer peptide conjugate for 4 h. Antigen presentation was assayed by measuring IL-2 produced after incubation with the T-cell line.
the endosomes into the cytosol. Depending on the exact peptide sequence, peptides may then be processed by the proteasome before being transported into the lumen of the ER by TAP, where the epitope binds to newly synthesized MHC class I molecules. The marked enhancement of presentation by incorporation of a 10-amino acid segment from the C terminus of DNA polymerase of herpes simplex virus (9) suggests that the Pol-loop segment may either facilitate rapid translocation from endocytic vesicles into the cytosol or protect the peptide from inappropriate cleavage events.

Our results on the influence of filipin and MCD on EtxB-mediated antigen presentation have established the site of release of the conjugated peptides from EtxB. The sterol-binding molecules filipin and MCD, by either disrupting or depleting cholesterol from cell membranes, can prevent the normal trafficking of Etx and the related cholera toxin to the Golgi/ER, without significantly affecting the ability of toxin to bind GM1 (20, 21, 37). In agreement with these previous findings we found that the transport of EtxB conjugates from early endosomes to the Golgi was inhibited by filipin and MCD. In the presence of filipin, EtxB localized with the early endosomal marker EEA1. Although we have not been able to demonstrate directly the cleavage of a substantial amount of conjugated peptide from EtxB-19-mer or -31-mer, the efficiency of EtxB-peptide conjugates to deliver epitopes into the MHC class I pathway was not affected by either filipin or MCD, suggesting that cleavage occurs in the endocytic compartment. The importance of endosomal acidification in permitting epitope delivery is demonstrated by the observation that BafA1 blocks antigen presentation in the presence of filipin.

Exogenous antigens can gain access to the proteasome and MHC class I pathway by several different routes (38). Recent studies by a number of groups have demonstrated antigen presentation after uptake by phagocytosis (39–41). For example, the phagocytosis of latex beads with antigen attached into macrophages can stimulate the proliferation of T cells. Antigen presentation following phagocytosis is partially inhibited by brefeldin A and is also dependent upon the TAP transporter (40). These results may be explained by the observation that the formation of phagosomes involves recruitment of the ER membrane (39–41) and this has the capacity to translocate peptides. TAP can occur in these membranes, and immunoproteasomes, that are normally enriched close to the ER (42), can also associate with phagosomes to facilitate processing of antigens delivered by this route (39). Our previous experiments have shown that EtxB does not enter cells by phagocytosis (43) and our observations suggest that the ability of peptides to translocate the endocytic membrane and avoid cleavage of the delivered epitope may depend on the unique properties of the conjugated peptides.

We investigated which protease(s) may be involved in peptide liberation from EtxB and in generating the mature epitope using protease inhibitors. Generation of epitopes may involve the action of several different proteolytic enzymes, depending on the sequence of the peptide delivered into the pathway. In some cases antigen processing is proteasome-dependent (reviewed in Refs. 2 and 3), whereas in others it requires the action of tryparedipeptidylpeptidase II (44, 45). In addition, peptides may be trimmed by cytosolic aminopeptidases such as leucine aminopeptidase, which is interferon-inducible, suggesting that it may play an important role in host defense (46). Leucine aminopeptidase has been demonstrated to be inhibited by bestatin, however, bestatin failed to have an effect on EtxB-mediated epitope delivery. Our results also indicate that the initial release of peptides from the EtxB is not catalyzed by cathepsin E, an endosomal aspartic proteinase that has been implicated in antigen processing (28), because the inhibitor pepstatin had no effect on antigen presentation. Our localization data suggests that EtxB conjugate entry into lysosomes is not required for efficient epitope presentation. This result is consistent with the observations that pepstatin and leupeptin, which are inhibitors of lysosomal cathepsin D and cysteine proteases, respectively, also have no effect on antigen presentation from EtxB conjugates. Our earlier observation that antigen presentation is inhibited in the presence of α-phenanthroline (9) may reflect the involvement of a metalloprotease but could also be explained by other effects on cells.

Although lactacystin and MG132 are not entirely specific for proteasomes (47), epoxomicin is believed to be specific for proteasomes at the concentrations used. Similar results were obtained with the JAWSII dendritic cells, E36.12.4 carcinoma cells, and RMA lymphoma cell lines. In each case delivery and presentation of SIINFEKL from the EtxB-31-mer, but not from the EtxB-19-mer, was inhibited by epoxomicin and other proteasome inhibitors implying that trimming of C-terminal amino acids (TEWTS) from the 31-mer peptide requires cleavage by the proteasome. Although it is well known that the proteasome is able to generate the correct C terminus of the SIINFEKL epitope (2, 13), we have demonstrated here that purified immunoproteasomes can also generate the authentic N terminus of the epitope. The lack of proteasome dependence of presentation from the EtxB-19-mer conjugate in cells is most likely because of the kinetics of proteasome-dependent cleavage and transport. The initial uptake of the EtxB conjugate into early endosomes is very rapid (9). However, the slow presentation of epitope from the 31-mer compared with the 19-mer in JAWSII cells suggests that proteasome-dependent cleavage of the 31-mer may be rate-limiting. Moreover, the rapid increase in presentation after the 20-min lag for the 31-mer as well as the rapid presentation from other peptides (19- and 26-mer) suggests that transport via TAP to the cell surface is much more rapid than access to and processing by the proteasome. This could explain why production of the epitope from the 19-mer can be proteasome-dependent in vitro but is proteasome-independent in cells. The length of the peptide transported into the ER may not be important because TAP is thought to be able to transport peptides as long as 40 amino acids (48). Once in the ER, epitopes extended on their N terminus could be trimmed by ERAP1 (49, 50) or other aminopeptidases before binding of the mature epitope to newly synthesized MHC class I molecules for transport to the cell surface.

Both the proteasome dependence of presentation from the 31-mer peptide EtxB conjugate and the TAP dependence of presentation from both 19-mer and 31-mer conjugates provide strong evidence that peptides containing the epitope are released directly into the cytosol. There does not appear to be any significant difference in the pathway of presentation from EtxB conjugates in different cell types, but this may depend on the peptide and the method of delivery (51). Once in the cytosol peptides may be degraded by peptidases such as thimet oligopeptidase (52), processed by the proteasome, or transported by TAP into the ER. There is a lot of evidence for the involvement of γ-interferon inducible proteasome subunits in antigen processing (2, 3) but it is not yet clear why proteasomes need to change their catalytic components in response to γ-interferon treatment. It is intriguing that the resulting immunoproteasomes are enriched in microsome preparations from rat liver and also at the ER in cultured cells (42, 53). In addition, it has been suggested that immunoproteasomes are able to generate longer peptide products more suited for antigen presentation (54). It is also of interest that 20 S proteasomes were able to generate the epitope from 31-mer in vitro but 26 S proteasomes...
were not. If these results can be extrapolated to the situation in cells, this observation suggests additional functions for 20 S proteasomes independent of 19 S regulatory complexes (55), possibly in association with the γ-interferon-inducible PA28 regulator.

In conclusion, our findings demonstrate that by combining the GM1 targeting function of EtxB, with the 10-amino acid Pol-loop segment of HSV-1 DNA polymerase, conjugated peptides can be rapidly delivered into the endogenous “classical” pathway of MHC class I presentation. Peptides are taken up into early endosomes, released, and transported directly into the cytosol. Depending on the choice of peptide and flanking sequences, antigen presentation may be either proteasome-dependent or not. Our observation that the epitope is presented more efficiently when derived from peptides that do not require proteasomal processing has important implications for future vaccine design.

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