Enhanced Delivery of Exogenous Peptides into the Class I Antigen Processing and Presentation Pathway

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Current immunization strategies, using peptide or protein antigens, generally fail to elicit cytotoxic-T-lymphocyte responses, since these antigens are unable to access intracellular compartments where loading of major histocompatibility complex class I (MHC-I) molecules occurs. In an attempt to circumvent this, we investigated whether the GM1 receptor-binding B subunit of *Escherichia coli* heat-labile toxin (EtxB) could be used to deliver class I epitopes. When a class I epitope was conjugated to EtxB, it was delivered into the MHC-I presentation pathway in a GM1-binding-dependent fashion and resulted in the appearance of MHC-I-epitope complexes at the cell surface. Importantly, we show that the efficiency of EtxB-mediated epitope delivery could be strikingly enhanced by incorporating, adjacent to the class I epitope, a 10-amino-acid segment from the C terminus of the DNA polymerase (Pol) of herpes simplex virus. The replacement of this 10-amino-acid segment by a heterologous sequence or the introduction of specific amino acid substitutions within this segment either abolished or markedly reduced the efficiency of class I epitope delivery. If the epitope was extended at its C terminus, EtxB-mediated delivery into the class I presentation pathway was found to be completely dependent on proteasome activity. Thus, by combining the GM1-targeting function of EtxB with the 10-amino-acid Pol segment, highly efficient delivery of exogenous epitopes into the endogenous pathway of class I antigen processing and presentation can be achieved.

Cytotoxic CD8⁺ T lymphocytes (CTLs) represent an important component of the protective and therapeutic immune response to intracellular bacteria, viruses, and tumors via their capacity to recognize foreign peptides that have bound to major histocompatibility complex class I (MHC-I) molecules (17, 32). The majority of the peptides presented are derived from endogenously synthesized proteins that are cleaved into small peptide fragments by the proteasome (reviewed in references 25 and 33). These are subsequently transported via the transporter of antigenic peptides into the lumen of the endoplasmic reticulum (ER), where they bind to newly synthesized MHC-I molecules. Such MHC-I peptide complexes are trafficked to the cell surface, whereupon they are recognized by T-cell receptors present on CTL precursors. This leads to CTL activation and subsequent CTL-mediated lysis of peptide-presenting cells.

Given the importance of CTLs in clearing the host of infected cells, there is great interest in the development of new vaccination strategies that are capable of inducing effective CTL responses. However, for vaccines composed of soluble protein antigens, immunization usually results in antigen uptake into an exogenous processing pathway that leads to peptide fragments being loaded onto MHC-II rather than MHC-I molecules (11, 23). Thus, in order for soluble antigens to induce MHC-I-restricted CTL responses, antigens need to access intracellular compartments where they can enter the endogenous class I processing and presentation pathway.

Bacterial protein toxins are molecules that combine

unique cell-binding properties with efficient cytosolic delivery properties (5). They would therefore appear to be ideally suited for the delivery of antigenic proteins and peptides in the class I presentation pathway, provided that detoxification without apparent loss of delivery capability can be achieved. Indeed, toxoid derivatives of the adenylate cyclase toxin of *Bordetella pertussis* (30), pertussis toxin (6), anthrax toxin (2, 10), and Shiga toxin B subunit (13) have been investigated as potential vehicles for delivery of peptides or proteins into the class I presentation pathway. The capacities of the nontoxic GM1-binding B subunits of *Escherichia coli* heat-labile toxin (EtxB) or cholera toxin (CtxB) to deliver antigens into the class I pathway have thus far not been investigated. However, researchers have recently shown that EtxB is capable of delivering peptides into specific intracellular compartments (18). In particular, when a 27-mer peptide derived from the C terminus of the DNA polymerase (Pol) of herpes simplex virus type 1 (HSV-1) was genetically fused to the C terminus of EtxB, it was found that the fusion protein entered cells and that the peptide was liberated from EtxB and translocated into the nuclear compartment. While structural features present in the Pol peptide were speculated to be involved in facilitating both its liberation from EtxB and its translocation from endosomal compartments, their contribution to peptide delivery remained undefined. Here, we have investigated (i) whether EtxB can be used as a vehicle for the delivery of exogenous peptides into the class I presentation pathway, (ii) whether EtxB-mediated peptide delivery is dependent on its capacity to bind to GM1 receptors, and (iii) whether incorporation of elements of the Pol peptide adjacent to the class I epitope would improve the efficiency of peptide delivery.

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TABLE 1. Peptides used in this study

Peptide	Sequence	Mass (Da)
8-mer	SIINFEKL	945
9-mer	CSIINFEKL	1,048
16-mer	CEKLAGFGSIINFEKL	1,751
19-mer	CAVGAGATAEESIINFEKL	1,905
19-mer ^{OVA}	CDEVSGLEQLESIINFEKL	2,198
26-mer	CEKLAGFGAVGAGATAEESIINFEKL	2,608
26-mer ^{EE→QQ}	CEKLAGFGAVGAGATAQQSIINFEKL	2,606
26-mer ^{V→R}	CEKLAGFGARGAGATAEESIINFEKL	2,665
31-mer	CEKLAGFGAVGAGATAEESIINFEKLTEWTS	3,212
9-mer ^{NP}	ASNENMETM	1,008
10-mer ^{NP}	CASNENMETM	1,111
20-mer ^{NP}	CAVGAGATAEEASNENMETM	1,968

MATERIALS AND METHODS

Production and characterization of EtxB and EtxB conjugates. Recombinant EtxB was expressed in a nontoxicogenic marine vibrio, *Vibrio* sp. strain 60, and purified using hydrophobic interaction and ion-exchange chromatography as reported earlier (1). A non-GM1-binding mutant of EtxB, EtxB(G33D), has been described previously (21) and was expressed and purified as described above. Purified preparations of EtxB and EtxB(G33D) were depleted of lipopolysaccharide using Detoxi-Gel columns (Pierce, Rockford, Ill.) and contained ≤ 50 endotoxin units per mg of protein, as determined in a *Limulus* amoebocyte lysate assay (BioWhittaker, Walkersville, Md.). Peptides were synthesized by solid-phase synthesis at the peptide synthesis unit of the Department of Biochemistry, University of Bristol, Bristol, United Kingdom, by G. Bloomberg. The peptides were purified by reverse-phase high-performance liquid chromatography, and their molecular masses were confirmed by mass spectrometry. The peptides synthesized contained well-characterized H-2^b-restricted MHC-I epitopes from either chicken egg ovalbumin (OVA), SIINFEKL (28), or influenza nucleoprotein (NP), ASNENMETM (27). The amino acid sequences and molecular weights of the peptides (in daltons) used in this study are listed in Table 1. For conjugation of peptides to EtxB, the chemical bifunctional cross-linker *N*-(gamma-maleimido-butyryl-oxy) succinimide (GMBS) (Pierce) was used. EtxB was first reacted with GMBS in a 1:4 molar ratio for 1 h at room temperature. Subsequently, excess GMBS was removed by gel filtration on a Sephadex G-25 column (Pharmacia, Uppsala, Sweden). Fractions containing EtxB-GMBS were pooled and reacted with peptide at a 1:2 molar ratio for 2 h at room temperature. Each peptide contained an N-terminal cysteine to allow direct reaction with the maleimide group in the GMBS molecule. After the conjugation reaction, unreacted GMBS groups were quenched by the addition of 2-mercaptoethanol (Sigma, Poole, United Kingdom) to a final concentration of 50 mM and incubation at room temperature for 30 min. Finally, EtxB conjugates were separated from excess peptide on a Sephadex G-50 column (Pharmacia). With all peptides, an EtxB pentamer/peptide ratio of approximately 1:5 was achieved, as determined by gel filtration on a Superdex 200 column connected to a SMART system (both from Pharmacia), using molecular weight standards. EtxB(G33D) peptide conjugates were generated under exactly the same conditions described above, and similar EtxB(G33D) pentamer/peptide ratios were obtained. Conjugates were analyzed either boiled or unboiled on sodium dodecyl sulfate (SDS)-polyacrylamide gels followed by staining with Coomassie brilliant blue R-250. The reactivity of conjugates with an EtxB pentamer-specific monoclonal antibody (MAb) (118-8; kindly provided by E. Lundgren and H. Persson, Department of Molecular Cell Biology, University of Umea, Sweden) was examined by Western blotting, and the GM1-binding properties of EtxB and of EtxB and EtxB(G33D) conjugates were assessed in a GM1 sandwich enzyme-linked immunosorbent assay (ELISA) essentially as previously described (1). The reactivity of conjugated peptides containing the SIINFEKL motif was assessed in a similar fashion using a SIINFEKL-specific polyclonal antiserum (a gift from Y. Reiss, Department of Biochemistry, Tel Aviv University, Tel Aviv, Israel).

Cell lines and culture conditions. JAWSII, an immortalized C57BL/6 bone marrow-derived dendritic cell line, was purchased from the American Type Culture Collection (Manassas, Va.) and cultured in RP10 medium (RPMI 1640 containing Glutamax-I, 100 μ g of penicillin-streptomycin/ml, and 10% fetal bovine serum [FBS] [all from GIBCO BRL, Paisley, United Kingdom]) supplemented with 2 ng of recombinant mouse granulocyte-macrophage colony-stim-

ulating factor (Sigma)/ml at 37°C in a humidified CO₂ incubator. The T-cell hybridomas RF33.70 and RF36.84 (24, 26), recognizing the OVA-derived SIINFEKL peptide or the NP-derived ASNENMETM peptide in the context of H-2^b, were a kind gift from K. L. Rock (University of Massachusetts Medical Center) and were cultured as described above in RP10 medium containing 20 mM HEPES, 1 mM nonessential amino acids, 25 μ M indomethacin, 0.25 μ g of amphotericin B (fungizone-1)/ml (all from GIBCO), and 50 μ M 2-mercaptoethanol.

Antigen presentation assays. Peptide presentation in MHC-I was examined by monitoring interleukin-2 (IL-2) release by the RF33.70 or RF36.84 T-cell hybridoma (24, 26). JAWSII dendritic cells were seeded in 96-well plates at 2×10^5 /ml and cultured overnight. The cells were then incubated with duplicate test samples at various concentrations and for various time intervals. In all experiments, equivalent amounts of either free or conjugated peptide were used. After incubation with antigen, the cells were fixed with 1% (wt/vol) paraformaldehyde in phosphate-buffered saline (PBS) for 10 min at room temperature, washed five times with medium, and incubated overnight with RF33.70 or RF36.84 T-cell hybridomas (5×10^5 cells/ml). Treatment of JAWSII cells with free 8-mer SIINFEKL or 9-mer^{NP} ASNENMETM peptide was used as a positive control, while PBS was used as a negative control. After overnight incubation, presentation-induced IL-2 secretion was determined using a commercially available IL-2 ELISA kit (Pharmingen, San Diego, Calif.). IL-2 levels are given as mean units per milliliter $\pm$ standard error of the mean (SEM). The data presented are representative of at least three independent experiments.

An alternative fluorescence-activated cell sorter (FACS)-based method for direct assessment of antigen presentation by JAWSII cells, involving the use of the 25D1.16 MAb directed against the MHC-I-SIINFEKL complex (22) (kindly donated by C. Reis e Sousa, Imperial Cancer Research Fund, London, United Kingdom, and R. N. Germain, National Institute of Allergy and Infectious Disease, National Institutes of Health, Bethesda, Md.) was also used to assess EtxB-mediated class I presentation. In brief, 2×10^6 to 4×10^6 JAWSII cells were treated with antigen at the equivalent concentration of 100 nM peptide for 2 h in a 25-cm² tissue culture flask. The cells were then trypsinized, centrifuged (5 min; $145 \times g$), washed with PBS-FBS-azide (PBS containing 5% FBS and 0.02% sodium azide), and incubated with 25D1.16 MAb (1:200; 30 min; 4°C). Subsequently, the cells were washed with PBS-azide and incubated with a fluorescein isothiocyanate (FITC)-labeled goat antibody specific for mouse immunoglobulin G (DAKO, Ely, United Kingdom) (1:500; 30 min; 4°C). Finally, the cells were washed with FACS flow (Becton Dickinson, San Jose, Calif.) and analyzed by flow cytometry (FACScan; Becton Dickinson). SIINFEKL peptide-treated and untreated cells were used as controls.

The inhibitory effects of bafilomycin A1 (BafA1), brefeldin A (BFA) (both from Sigma), and epoxomicin (Calbiochem, Nottingham, United Kingdom) on EtxB-mediated epitope delivery were also studied. In such experiments, JAWSII cells were pretreated with inhibitors for 1 h at various concentrations and subsequently incubated with EtxB conjugates or EtxB and peptide alone or admixed for 2 h and processed as described above.

Confocal microscopy. For microscopic analysis, JAWSII cells were first grown on sterile coverslips coated with rat collagen type II (Sigma) for 48 h. Subsequently, the cells were treated for various periods of time with EtxB conjugates, fixed with 4% (vol/vol) paraformaldehyde in PBS for 10 min, and then permeabilized by incubation in 4% paraformaldehyde containing 0.5% Triton X-100 (Sigma) for 15 min. After repeated washing with PBS, the cells were incubated

with either MAb 25D1.16, specific for the MHC-I-SIINFEKL complex (1:200), or an EtxB-specific polyclonal rabbit antiserum (1:500) (kindly provided by M. Pizza, IRIS, Siena, Italy) diluted in PBS-bovine serum albumin (PBS containing 3% bovine serum albumin [fraction V; Sigma]) for 1 h at room temperature. The cells were then washed with PBS and incubated with FITC- or tetramethyl rhodamine isothiocyanate-labeled secondary antibodies directed against either mouse or rabbit immunoglobulin G (1:100) (Jackson Immuno Research Laboratories, West Grove, Pa.). In some experiments, fixed cells were pretreated with rhodamine-labeled wheat germ agglutinin (WGA; Sigma) to visualize plasma and Golgi membranes. The washed coverslips were then mounted on glass examination slides spotted with Mowiol containing 2.5% 1,4-diazabicyclo[2.2.2] octane antifading and 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) (1 mg/ml) for nuclear staining (all from Sigma) and then examined using a DH1RBE inverted confocal microscope (Leica, Buffalo, N.Y.) at the Medical Research Council Cell Imaging Facility of the Department of Biochemistry, University of Bristol.

RESULTS

Epitope attachment to EtxB. To investigate whether EtxB could be used as a vehicle for the delivery of peptides into the class I presentation pathway, we initially designed two peptides, a 9-mer and a 26-mer peptide, both containing the well-characterized class I epitope (SIINFEKL) of OVA. The 9-mer peptide comprised the SIINFEKL epitope and an N-terminal cysteine to allow conjugation to EtxB (Table 1). The design of the 26-mer peptide was based on a previous study, in which it was shown that a 27-mer derived from the Pol of HSV-1, when fused to the C terminus of EtxB, was efficiently delivered into eukaryotic cells (18). Since the Pol peptide contained a number of features speculated to be involved in peptide liberation and endosomal translocation, namely, a putative cathepsin D cleavage site (EKL ↓ AG ↓ F) and a loop segment of hydrophobic and charged amino acids (AGFGAVGAGATAEE), these elements were incorporated adjacent to the SIINFEKL epitope in order to investigate whether they would exert an effect on the efficiency of peptide delivery. Thus, a 26-mer synthetic peptide was designed containing an N-terminal cysteine residue suitable for chemical conjugation, the putative cleavage site and loop segment from the Pol peptide, and the SIINFEKL epitope (Table 1).

The 9-mer and 26-mer peptides were chemically conjugated to EtxB using the bifunctional cross-linker GMBS as described in Materials and Methods. The peptides were also conjugated to a well-characterized mutant of EtxB, EtxB(G33D), which retains the structural and biophysical characteristics of native EtxB but lacks affinity for GM1 (20, 21). The resultant conjugates retained the characteristic stability properties of EtxB, migrating as a pentameric high-molecular-weight species on SDS-polyacrylamide gels if kept unheated prior to analysis (Fig. 1A, lanes 1, 3, 5, and 7) and dissociating into monomers when boiled (Fig. 1A, lanes 2, 4, 6, and 8). The unheated conjugates had an electrophoretic mobility that was slower and had a more diffuse appearance than that of the native EtxB pentamer, suggestive of attachment of several peptides per pentamer (Fig. 1A; compare lane 9 with lanes 1, 3, 5, and 7). Upon boiling, monomeric conjugate species with one, two, or more conjugated peptides per EtxB monomer could be clearly distinguished in the EtxB-26-mer and EtxB(G33D)-26-mer conjugates (Fig. 1A, lanes 6 and 8). In the case of the EtxB-9-mer and EtxB(G33D)-9-mer conjugates containing one, two, or more peptides, the monomeric conjugate species were clustered with similar electrophoretic mobilities (Fig. 1A, lanes 2

FIG. 1. Production and characterization of EtxB- and EtxB(G33D)-9-mer and -26-mer conjugates. (A) SDS-PAGE analysis of EtxB- and EtxB(G33D)-9-mer and -26-mer conjugates. Lanes: 1, EtxB-9-mer, unheated; 2, EtxB-9-mer boiled; 3, EtxB(G33D)-9-mer, unheated; 4, EtxB(G33D)-9-mer, boiled; 5, EtxB-26-mer, unheated; 6, EtxB-26-mer, boiled; 7, EtxB(G33D)-26-mer, unheated; 8, EtxB(G33D)-26-mer, boiled; 9, EtxB, unheated; 10, EtxB, boiled. Molecular mass standards in kilodaltons and the EtxB monomer and pentamer (arrows) are indicated. (B and D) Detection of EtxB- and EtxB(G33D)-9-mer and -26-mer conjugates in GM1 ELISA using a MAb specific for EtxB (118-8). (C and E) Detection of EtxB- and EtxB(G33D)-9-mer and -26-mer conjugates in GM1 ELISA using a SIINFEKL-specific polyclonal antiserum. Absorbances are plotted against the dilution factor and are given as means $\pm$ SEM.

and 4), presumably due to the lower molecular weight of the conjugated peptide. In GM1-binding ELISAs, the EtxB-9-mer and -26-mer conjugates could be readily detected by MAb 118-8, specific for the EtxB pentamer, confirming that the coupling procedure had not interfered with the capacity of the EtxB moiety of the conjugates to bind to GM1 (Fig. 1B and D). By contrast, the EtxB(G33D) conjugates were not detectable in the GM1 ELISA, reflecting the inability of the EtxB(G33D) mutant to bind to GM1 (Fig. 1B and D). The use of a SIINFEKL-specific antiserum demonstrated that the EtxB-9-mer and EtxB-26-mer conjugates display the epitope when the conjugates are bound to GM1 (Fig. 1C and E). Western blot analysis using MAb 118-8 detected both native EtxB and the EtxB and EtxB(G33D) conjugates, while use of the SIINFEKL-specific antiserum detected both the pentameric and monomeric species of the conjugates (data not shown). For all conjugates, the peptide/EtxB pentamer ratio, estimated

by gel filtration chromatography, together with the conjugate concentration, was used to determine the apparent concentration of peptide in the conjugate as described in Materials and Methods. On average, with each peptide, an EtxB pentamer/peptide ratio of approximately 1:5 was achieved.

EtxB conjugates deliver SIINFEKL peptide into the MHC-I presentation pathway in a dose- and GM1-binding-dependent fashion. The capacities of the EtxB- and EtxB(G33D)-9-mer and -26-mer conjugates to deliver the OVA-derived SIINFEKL epitope into MHC-I were investigated in antigen presentation assays using JAWSII cells as antigen-presenting cells and IL-2 release by the SIINFEKL-specific RF33.70 T-cell hybridoma as a read-out for antigen presentation. In these assays, the free 8-mer SIINFEKL peptide was used as a positive control. This peptide is able to access the MHC-I peptide-binding groove directly, by competing for binding with peptides present in already-displayed MHC-I complexes. Figure 2A shows that the free 9-mer SIINFEKL peptide also stimulated class I presentation, indicating that, like the 8-mer, the 9-mer peptide is capable of loading directly onto MHC-I molecules present on the cell surface. When the 9-mer was conjugated to EtxB and the resultant conjugate was tested at concentrations ranging from 1 pM to 100 nM peptide equivalents, it was found that the SIINFEKL epitope was delivered in a dose-dependent fashion (Fig. 2A). However, the observed extent of epitope presentation was not as great as that achieved with equivalent amounts of free 8-mer or 9-mer peptide (Fig. 2A). Importantly, treatment of JAWSII cells with equivalent concentrations of the EtxB(G33D)-9-mer conjugate failed to result in epitope presentation, indicating that delivery is dependent on GM1 binding. By comparison, the EtxB-26-mer conjugate was much more efficient at delivering the SIINFEKL epitope than the corresponding EtxB-9-mer conjugate (Fig. 2B). Moreover, delivery of the SIINFEKL epitope by the EtxB-26-mer conjugate was dose dependent and reached maximal levels at concentrations of 10 nM peptide equivalents, similar to that observed when using the free 8-mer SIINFEKL peptide (Fig. 2B). Importantly, no presentation occurred when either the free 26-mer peptide or the EtxB(G33D)-26-mer conjugate was tested, indicating that conjugation to a functional, GM1-binding EtxB moiety is essential for the 26-mer to be delivered into the class I pathway. The striking difference in the extent of SIINFEKL presentation mediated by the EtxB-9-mer and EtxB-26-mer conjugates implies that N-terminal Pol-derived segments within the 26-mer conjugate are exerting an influence on the efficiency of epitope presentation.

For a more direct assessment of antigen presentation, a FACS-based assay involving the use of MAb 25D1.16, specific for MHC-I-SIINFEKL complexes, was employed. The results, obtained at 100 nM peptide equivalents, were in complete agreement with the data obtained in antigen presentation assays as described above (Fig. 2C). Accordingly, the EtxB-26-mer conjugate and free 8-mer SIINFEKL peptide induced clear and similar shifts, while EtxB-9-mer induced a modest shift in fluorescence. The free 8-mer and 9-mer peptides displayed very similar shifts, while 26-mer peptide alone, admixed with EtxB, or conjugated to EtxB(G33D) failed to induce a shift in fluorescence (data not shown). The observed enhancement of antigen presentation was not due to EtxB-induced upregulation of MHC-I expression, as FACS analysis using

FIG. 2. EtxB conjugates deliver SIINFEKL epitopes into the MHC-I presentation pathway in a dose- and GM1-binding-dependent fashion. (A and B) EtxB-induced antigen presentation as assessed by analysis of IL-2 release by RF33.70 T-cell hybridoma. JAWSII cells were incubated with peptide alone or EtxB- or EtxB(G33D)-peptide conjugates at the indicated equimolar concentrations of peptide for 2 h. The cells were then fixed with 1% paraformaldehyde and incubated overnight with RF33.70 cells; 8-mer peptide and PBS were used as the positive and negative control, respectively. Duplicate samples were tested, and the data are given as means $\pm$ SEM. (A) 9-mer peptide and EtxB- and EtxB(G33D)-9-mer conjugates. (B) 26-mer peptide and EtxB- and EtxB(G33D)-26-mer conjugates. (C) EtxB-induced antigen presentation as assessed by FACS analysis using MAb 25D1.16, specific for MHC-I-SIINFEKL complexes. JAWSII cells were treated with 100 nM 8-mer peptide (grey curve), EtxB-9-mer conjugate (dashed curve), EtxB-26-mer conjugate (solid curve), or PBS (filled curve) for 2 h and then sequentially incubated with 25D1.16 MAb and a FITC-labeled secondary antibody. Fluorescence was assessed by flow cytometric analysis.

FITC-labeled MHC-I-specific antibodies did not reveal a significant increase in cell fluorescence (data not shown). Thus, the observed IL-2 release was the result of the appearance of MHC-I-SIINFEKL complexes on the cell surface and subsequent recognition and IL-2 production by the RF33.70 T-cell hybridoma.

The Pol loop segment is responsible for the increase in the efficiency of EtxB-mediated epitope delivery. In an attempt to identify which of the Pol peptide-derived structural elements within the 26-mer peptide were responsible for facilitating more efficient epitope delivery, two truncated peptides, namely, a 16-mer and a 19-mer peptide, were designed (Table 1). Compared to the 26-mer peptide, the 16-mer peptide lacked the Pol loop segment, while the 19-mer peptide lacked the putative cathepsin cleavage site. The 16-mer and 19-mer peptides were conjugated to EtxB as described above, and the

FIG. 3. The Pol loop segment enhances the efficiency of EtXB-mediated peptide delivery. (A) Effect of peptide truncation on EtXB-mediated epitope delivery. JAWSII cells were incubated with the indicated peptides alone or peptides admixed with or conjugated to EtXB at the equivalent concentration of 10 nM peptide for 2 h. The cells were then fixed with paraformaldehyde, and antigen presentation was assessed by determining antigen presentation-induced IL-2 release by RF33.70 T cell hybridoma. (B) Kinetics of antigen presentation upon treatment of JAWSII cells with EtXB-9-mer, -16-mer, -19-mer, or -26-mer conjugate. JAWSII cells were incubated for 0, 10, 15, 30, 60, or 120 min and fixed with paraformaldehyde, and antigen presentation was assessed as described above. Duplicate samples were tested, and data are given as means $\pm$ SEM.

abilities of the resultant conjugates to bind to GM1 were confirmed by GM1 sandwich ELISA (data not shown). Figure 3A shows that, at 10 nM peptide equivalents, the free 16-mer, 19-mer, and 26-mer peptides could not load directly onto MHC-I. When the EtXB-9-mer and EtXB-16-mer were tested under these conditions, neither conjugate was able to deliver sufficient SIINFEKL epitope to stimulate IL-2 release by the RF33.70 T-cell hybridoma, indicating that the inclusion of the putative cathepsin D cleavage site is not responsible for the enhanced epitope delivery observed with the EtXB-26-mer. By contrast, conjugation to EtXB of the 19-mer, which contains the Pol loop segment, resulted in high-level peptide delivery, comparable to the maximal loading achieved with free 8-mer SIINFEKL peptide (Fig. 3A). We therefore conclude that incorporation of the Pol loop segment adjacent to the class I epitope causes a marked increase in the extent of EtXB-mediated epitope presentation.

To assess the kinetics of appearance of MHC-I-SIINFEKL complexes on the cell surface upon treatment with EtXB-9-mer, -16-mer, -19-mer, and -26-mer conjugates, cells were fixed at various time points after incubation with the EtXB conjugates. After 0 min of incubation with the conjugates, no peptide presentation was evident. Remarkably, after 15 min, maximal presentation levels had already been attained by the EtXB-19-mer and -26-mer conjugates, which displayed very similar kinetics (Fig 3B). In agreement with the findings described above, no significant levels of presentation were observed with the EtXB-9-mer and -16-mer conjugates, even after 120 min.

FIG. 4. Mutation or replacement of the Pol segment reduces efficiency or inhibits EtXB-mediated epitope delivery. (A) Effect of amino acid substitution or Pol segment replacement on the extent of epitope delivery. (B) Kinetics of antigen presentation upon treatment of JAWSII cells with EtXB-19-mer, -19-mer^{OVA}, -26-mer^{EE→QQ}, -26-mer^{V→R}, or -31-mer conjugate. For details, see the legend to Fig. 3. Duplicate samples were tested, and the data are given as means $\pm$ SEM.

To further investigate whether the intrinsic properties of the Pol loop segment contributed to peptide delivery, three additional peptides were designed (Table 1). A 26-mer^{EE→QQ} and a 26-mer^{V→R} peptide were designed to assess the effects of targeted mutations in the Pol loop segment on delivery. These peptides contained either two Glu-to-Gln substitutions, which alter both the overall charge of the peptide and the potential cleavage site N-terminal to the SIINFEKL epitope, or a single Val-to-Arg substitution, which should disrupt the relative hydrophobicity of the Pol loop segment (Table 1). In addition, a further 19-mer peptide, termed 19-mer^{OVA}, was prepared in which the 10-amino-acid Pol loop segment was replaced by the 10 amino acids that normally precede the SIINFEKL epitope in native OVA. This peptide permitted us to investigate if the observed increase in epitope delivery was due to the intrinsic properties of the Pol loop segment or simply due to peptide length. The three peptides were conjugated to EtXB as described above, and the abilities of the resultant conjugates to bind to GM1 were confirmed by GM1 sandwich ELISA (data not shown).

When these peptides were tested alone or admixed with EtXB at 10 nM peptide equivalents, none of them were efficiently presented. However, upon conjugation, the EtXB-19-mer^{OVA}, -26-mer^{EE→QQ}, and -26-mer^{V→R} conjugates displayed markedly altered epitope delivery characteristics compared with EtXB-19-mer (Fig 4A). The EtXB-19-mer^{OVA} and -26-mer^{EE→QQ} conjugates were found to almost completely lack the ability to stimulate epitope presentation, and the EtXB-26-mer^{V→R} conjugate displayed a reduction in the extent of epitope presentation (Fig. 4A). Furthermore, the V→R substitution resulted in a marked alteration in the kinetics of SIINFEKL epitope delivery, with no presentation evident in the first 15 min and presentation still rising at 120

min (Fig. 4B). We conclude, therefore, that the Pol loop segment possesses an intrinsic ability to efficiently deliver peptides and that the enhanced extent of epitope presentation observed with the original EtxB-19-mer and -26-mer conjugates is not simply due to the length of the peptide preceding the SIINFEKL epitope.

Endosomal acidification and an intact Golgi are required for EtxB-mediated epitope delivery. The trafficking pathway by which EtxB mediates the delivery of conjugated peptides into the MHC-I pathway was investigated using BafA1, an inhibitor of the V-ATPase responsible for acidification of organelles of the endocytic pathway (3), and BFA, a Golgi-disrupting drug and inhibitor of vesicle-mediated secretion (16). Treatment of JAWSII cells for 60 min with BafA1 or BFA, prior to addition of the EtxB-19-mer, -26-mer, and -26-mer^{V→R} conjugates, led to complete inhibition of EtxB-mediated epitope delivery, as assessed by both the IL-2 release assay and FACS detection of MHC-I-SIINFEKL complexes. Since the results obtained with all of these conjugates were identical, only the data using the EtxB-19-mer are shown (Fig. 5B, C, F, and G). Importantly, treatment of JAWSII cells with BafA1 or BFA did not inhibit the direct loading and presentation of the free 8-mer peptide (Fig. 5B-C). Also, when ammonium chloride or monensin, an Na⁺-ionophor inhibitor of endosomal acidification, was tested, EtxB-mediated epitope delivery was prevented while presentation of the free 8-mer peptide was unaffected (data not shown). In other studies, inhibitors of acid proteases, metalloaminopeptidases, and serine and cysteine proteases (pepstatin, bestatin, and leupeptin, respectively) were found not to have a significant effect on EtxB-mediated epitope presentation (data not shown). However, the metalloprotease inhibitor 1,10-phenanthroline was found to inhibit EtxB-induced antigen presentation (data not shown). Taken together, these findings suggest that EtxB-mediated peptide presentation depends upon (i) conjugate entry into acidic endosomes; (ii) peptide cleavage, possibly by a metalloprotease; and (iii) a functionally intact Golgi network and secretory pathway.

Proteasome involvement in EtxB-mediated epitope presentation. To assess the possible requirement for proteasome-mediated processing of peptides delivered by EtxB, we designed an additional peptide, namely, a 31-mer. The design of this peptide was based on studies in which it was shown that proteasome cleavage of OVA creates the proper C terminus of the SIINFEKL epitope whereas distinct peptidases in the cytosol or ER generate the appropriate N terminus from extended peptides (7, 8). Consequently, since all of the peptides we had tested contained the SIINFEKL epitope at their C termini, it is highly unlikely that the pathway of delivery of these peptides would depend on proteasome-mediated cleavage. We therefore extended the 26-mer peptide at the C terminus with an additional five amino acids derived from the OVA sequence, thus creating an internal SIINFEKL epitope (Table 1), and tested the effects of BafA1, BFA, and a number of well-characterized proteasome inhibitors on peptide delivery.

Incubation of JAWSII cells with the EtxB-31-mer resulted in the efficient presentation of the SIINFEKL epitope, as assessed by the IL-2 release assay and by FACS (Fig. 5A and E). As observed with the EtxB-19-mer conjugate, prior treatment with BafA1 or BFA prevented EtxB-31-mer-mediated epitope

FIG. 5. Effects of inhibitors on EtxB-induced antigen presentation. The effects of BafA1, BFA, and epoxomicin on delivery of 19-mer and 31-mer peptides were assessed by both IL-2 release assays (A to D) and FACS analysis using the 25D1.16 antibody (E to H). PBS and the 8-mer peptide were used as positive and negative controls, respectively. IL-2 release data are given as means $\pm$ SEM. In panels E to H, EtxB-19-mer (solid curve), EtxB-31-mer (dashed curve), and PBS (filled curve) are shown. For details, see the legend to Fig. 2. (A and E) Antigen presentation upon treatment with 19-mer or 31-mer peptide alone, admixed with EtxB, or conjugated with EtxB in the absence of inhibitors. (B and F) Effect of BafA1 (200 nM). (C and G) Effect of BFA (10 μ M). (D and H) Effect of epoxomicin (10 μ M).

presentation (Fig. 5B, C, F, and G). In agreement with the notion that the proteasome does not generate the N terminus of the SIINFEKL epitope, preincubation of JAWSII cells with epoxomicin, a specific proteasome inhibitor (19), did not inhibit epitope presentation when the cells were treated with EtxB-19-mer or free 8-mer (Fig. 5D and H). Likewise, lactacystin or MG132, two additional inhibitors of proteasome activity, failed to prevent EtxB-mediated or free-epitope presentation (data not shown). Similar results were obtained when all of the other EtxB peptide conjugates were tested in the presence of epoxomicin, lactacystin, or MG132 (data not shown). However, in contrast to the behavior of the conjugates described above, epitope delivery by the EtxB-31-mer was completely inhibited by the addition of epoxomicin (Fig. 5D and H). Similar inhibition was observed when lactocystin and MG132 were used (data not shown). This demonstrates that proteasome-mediated cleavage of the 31-mer peptide is necessary for it to enter the class I presentation pathway.

To further visualize the trafficking pathway of the EtxB conjugates and to determine the localization of MHC-I complexes, cells were treated with EtxB-19-mer or EtxB-31-mer and probed with antibodies directed against EtxB or MHC-I-SIINFEKL and then examined by confocal microscopy. After 1 min of incubation with the conjugates, the EtxB moiety could be clearly seen at the cell surface and there was no evidence of detectable MHC-I-SIINFEKL complexes (Fig.

6A, images e to h). After 120 min, both EtxB-19-mer and -31-mer were almost completely internalized, and perinuclear staining was evident with both anti-EtxB and anti-MHC-I-SIINFEKL antibodies, with considerable colocalization (Fig. 6A, images i to l).

This perinuclear staining was suggestive of localization of both EtxB and the MHC-I-SIINFEKL complexes in the ER or Golgi network, consistent with both the trafficking pathway of EtxB (14) and the normal cellular location of newly synthesized MHC-I molecules. In order to identify the cellular localization of the MHC-I-SIINFEKL complexes more accurately, fixed cells were treated with rhodamine-labeled WGA, specific for *N*-acetyl- β -D-acetylglucosamine present in Golgi-ER and plasma membranes (31), followed by anti-MHC-I-SIINFEKL and secondary antibodies (Fig. 6B). It was found that WGA and MHC-I-SIINFEKL complexes colocalized, confirming that these complexes were present in the Golgi (Fig. 6B, images a to d). Moreover, when cells were preincubated with epoxomicin to inhibit proteasome activity, no staining with MHC-I-SIINFEKL-specific antibodies was obtained when the cells were treated with EtxB-31-mer (Fig. 6B, images d and h), whereas normal colocalization of WGA and MHC-I-SIINFEKL complexes was observed when cells were treated with the EtxB-19-mer (Fig. 6B, images c and g). In addition, no detectable MHC-I-SIINFEKL complexes were observed when cells were pretreated with BafA1 or BFA prior to addition of the EtxB-19-mer or EtxB-31-mer conjugate (data not shown). The above-mentioned findings on the effects of the trafficking and proteasome inhibitors are in full agreement with the results obtained in the antigen presentation assays and indicate that peptides are delivered into the endogenous antigen processing and presentation pathway.

EtxB-mediated delivery of a class I epitope from influenza NP is also enhanced by the Pol loop segment. To establish if conjugation of Pol loop segment-containing peptides to EtxB represents a generic approach for enhanced delivery of class I epitopes into the endogenous pathway, two additional peptides containing a well-characterized H-2^b-restricted epitope from influenza NP were designed. A 10-mer peptide, 10-mer^{NP}, comprising the NP-derived ASNENMETM epitope and an N-terminal cysteine for conjugation to EtxB, and a 20-mer peptide, 20-mer^{NP}, comprising the ASNENMETM epitope, an N-terminal cysteine residue, and the 10-amino-acid Pol loop segment, were synthesized (Table 1). The 10-mer^{NP} and 20-mer^{NP} peptides were conjugated to EtxB as described above, and the abilities of the resultant conjugates to bind to GM1 were confirmed by GM1 sandwich ELISA (data not shown). Similarly, peptides were conjugated to EtxB(G33D), and the conjugates were found to lack GM1-binding activity (data not shown).

The capacities of the EtxB- and EtxB(G33D)-10-mer^{NP} and -20-mer^{NP} conjugates to deliver the NP-derived ASNENMETM epitope into MHC-I were investigated in antigen presentation assays using JAWSII cells as antigen-presenting cells and IL-2 release by the RF36.84 T-cell hybridoma as a read-out for antigen presentation. The results, shown in Fig. 7, were in complete agreement with those obtained using the peptides containing the SIINFEKL epitope. Accordingly, both the 9-mer^{NP} and 10-mer^{NP} peptide were found to be capable of displacing peptides bound to cell surface MHC-I molecules, resulting in maximal

FIG. 6. MHC-I-SIINFEKL complexes are located in the Golgi compartment. (A) Confocal microscopic analysis of the cellular localization of EtxB and MHC-I-SIINFEKL complexes upon treatment of JAWSII cells with EtxB-19-mer and EtxB-31-mer. JAWSII cells were incubated for 1 min (images e to h) or 2 h with either EtxB-19-mer or EtxB-31-mer conjugate (images i to l) or PBS (images a to d). After treatment, the cells were fixed with paraformaldehyde and then stained with a polyclonal rabbit antiserum specific for EtxB and a MAb specific for MHC-I-SIINFEKL-complexes (25D1.16), followed by FITC- or rhodamine-labelled secondary antibodies as described in Materials and Methods. Cell nuclei were stained with DAPI (blue). For EtxB-31-mer, only the overlay is shown. (B) Colocalization of MHC-I-SIINFEKL complexes and Golgi membranes. Cells were treated as described above and incubated with rhodamine-labeled WGA, 25D1.16 MAb, and secondary antibodies (images a to d). The effect of the proteasome inhibitor epoxomicin (10 μ M) on formation of MHC-I-SIINFEKL complexes is also shown (images e to h).

stimulation of epitope presentation. When the 10-mer^{NP} peptide was conjugated to EtxB and the resultant conjugate was tested at concentrations ranging from 1 to 100 nM peptide equivalents, it was found that significant ASNENMETM epitope presentation was obtained only at 100 nM peptide equivalents (Fig. 7A). Similar to earlier observations, the extent of epitope presentation was not as great as that achieved with equivalent amounts of free 9-mer^{NP} or 10-mer^{NP} peptide but was dependent on GM1 binding, as treatment of JAWSII cells with the EtxB(G33D)-10-mer^{NP} conjugate at 100 nM peptide equivalents failed to result in epitope presentation (Fig. 7A). Finally, compared to treatment of JAWSII cells with the EtxB-10-mer^{NP} conjugate, treatment with the EtxB-20-mer^{NP} conjugate resulted in much higher levels of ASNENMETM epitope presentation, indicating that inclusion of the Pol loop segment facilitated more efficient delivery of

FIG. 7. The Pol loop segment enhances EtxB-mediated delivery of an influenza NP-derived class I epitope. (A and B) EtxB-induced antigen presentation as assessed by analysis of IL-2 release by RF36.84 T-cell hybridoma. JAWSII cells were incubated with peptide alone or EtxB- or EtxB(G33D)-peptide conjugates at the indicated equimolar concentrations of peptide for 2 h. The cells were then fixed with 1% paraformaldehyde and incubated overnight with RF36.84 cells; the 9-mer^{NP} peptide and PBS were used as the positive and negative control, respectively. Duplicate samples were tested, and the data are given as means $\pm$ SEM. (A) 10-mer^{NP} peptide and EtxB- and EtxB(G33D)-10-mer^{NP} conjugates. (B) 20-mer^{NP} peptide and EtxB- and EtxB(G33D)-20-mer^{NP} conjugates.

the NP-derived peptide (Fig. 7B). Importantly, no presentation occurred when either the free 20-mer^{NP} peptide or the EtxB(G33D)-20-mer^{NP} conjugate was tested, indicating that conjugation to a functional, GM1-binding EtxB moiety is essential for the 20-mer^{NP} to be delivered into the class I pathway. We therefore conclude that conjugation of peptides to EtxB facilitates their entry into the cell via a GM1-binding-dependent pathway and that the efficiency of EtxB-mediated delivery can be strikingly enhanced by the inclusion of the Pol loop segment in the conjugated peptide, leading to highly efficient delivery of the epitope into the endogenous class I antigen processing and presentation pathway.

DISCUSSION

Bacterial A-B protein toxins are composed of structurally and functionally distinct enzymatically active A and receptor-binding B subunits or domains, of which the A domain requires enzymatic cleavage and entry into the target cell cytosol in order for it to exert its toxic enzymatic effects (for a review, see reference 5). Given the intrinsic capacities of these toxins to deliver their toxic A subunits into cells, several investigators have attempted to exploit A-B toxins for the delivery of heterologous antigens into the endogenous class I pathway. Most of these studies have focused on the development of recombinant constructs comprising heterologous peptide or protein antigens fused to the toxin A subunits. Such constructs have been shown to be capable of stimulating MHC-I-restricted presentation of the heterologous antigen (2, 6, 30). The use of

the toxin B subunits of bacterial toxins as delivery vehicles has not been widely evaluated because, in contrast to the A subunits, the toxin B subunits are not thought to enter the cell cytosol. However, Lee et al. showed that fusion of a peptide epitope to the B subunit of Shiga toxin resulted in presentation of the epitope in the context of MHC-I (13). Here, we report the systematic evaluation of the use of the receptor-binding B subunit of *E. coli* heat-labile toxin as a vehicle for the delivery of peptide epitopes into the class I pathway. We chose to adopt a versatile chemical conjugation strategy rather than a genetic strategy for attaching peptides to EtxB in order to facilitate the rapid preparation of conjugates containing different peptides. We show for the first time that EtxB can be used for the intracellular delivery of the well-characterized SIINFEKL class I epitope from OVA and that EtxB-mediated delivery is dependent on its ability to bind to GM1 receptors on the target cell. However, conjugation of the SIINFEKL epitope alone to EtxB, as exemplified by the EtxB-9-mer conjugate, resulted in inefficient presentation relative to direct loading with the free 8-mer SIINFEKL epitope.

In an attempt to overcome this, we sought to take advantage of our previous observation that a 27-mer peptide derived from the C terminus of the Pol peptide of HSV-1 was efficiently delivered into intracellular compartments when fused to EtxB, a finding that suggested that the Pol peptide may contain a number of features which facilitate both liberation from EtxB and translocation from endosomal compartments. These included a putative cathepsin D cleavage site and a loop segment of hydrophobic and charged amino acids. We therefore decided to investigate whether incorporation of these elements adjacent to the SIINFEKL epitope would improve the efficiency of EtxB-mediated peptide delivery into the class I pathway. We found that the inclusion of elements of the Pol peptide in the conjugated peptides contributed to the extent and efficiency of epitope presentation. In this respect, such EtxB-Pol peptide-SIINFEKL conjugates were capable of achieving levels of presentation comparable to those resulting from direct loading by the free SIINFEKL peptide. We were able to map the region of the Pol peptide necessary for efficient delivery to a 10-amino-acid region corresponding to the Pol loop segment. This segment is part of a 36-amino-acid hairpin-like structure, consisting of two helical regions interrupted by a flexible loop region that contains two glutamate residues (4, 9). The Pol segment used in the present study contains the two glutamates and the flexible region composed of hydrophobic and nonpolar amino acids and shows a degree of similarity with fusion peptides from viral glycoproteins (29). Therefore, one explanation for the improved delivery of the SIINFEKL epitope by peptides containing the Pol loop segment may be that this segment has an intrinsic propensity to penetrate lipid bilayers. Furthermore, it is known that for pH-dependent translocation of diphtheria toxin, protonation of acidic residues in helical hairpins permits insertion of hydrophobic domains into lipid bilayers, and mutation of such residues in diphtheria toxin is known to result in a reduction in toxicity (12). Thus, liberation from EtxB, followed by protonation of the glutamates and then translocation across a vesicular membrane into the cytosol, should permit highly efficient entry into the endogenous class I presentation pathway.

In support of this hypothesis, replacement of the 10-amino-

FIG. 8. Schematic representation of the pathway of EtxB-mediated delivery of class I epitopes. GM1 binding by the EtxB-peptide conjugate mediates uptake into smooth endocytic vesicles. Upon endosomal acidification, the peptide is cleaved from the conjugate and translocated into the cytosolic compartment, while EtxB traffics to the trans-Golgi network (TGN). Depending on the exact peptide sequence, the peptide is processed by the proteasome and, following transport into the lumen of the ER by the transporter of antigenic peptides (TAP), binds to empty MHC-I molecules. Stable MHC-I-peptide complexes are then transported to the cell surface.

acid Pol loop segment with the 10 amino acids that precede SIINFEKL in native OVA completely abolished the enhancement of epitope presentation observed with the EtxB-19-mer conjugate. Furthermore, conjugation to EtxB of a 26-mer^{EE→QQ} peptide, in which the two Glu residues present in the native 26-mer peptide are replaced by Gln residues, or a 26-mer^{V→R} peptide, in which the stretch of nonpolar and hydrophobic amino acid residues in the Pol loop segment is

interrupted by an Arg residue, resulted in a marked decrease in the efficiency of epitope delivery, possibly due to decreased translocation efficiency. Moreover, the finding that BafA1, ammonium chloride, and monensin inhibited EtxB-mediated epitope presentation indicates that entry into an acidic endosome is essential. Given that the trafficking and toxicity of cholera toxin is refractory to chaotropic agents (15), this would imply that entry into an acidic environment is required for efficient epitope delivery rather than for trafficking of the carrier. Consequently, an acidic environment could enable protonation of the Pol loop glutamate residues for subsequent translocation.

It is also possible that entry into acidic endosomes is necessary for peptide liberation from EtxB as a result of the activities of acid-dependent proteases, such as cathepsins. However, when EtxB-mediated presentation of the 26-mer peptide was assessed in the presence of pepstatin, an inhibitor of acid proteases, it had no effect on the extent of SIINFEKL presentation (data not shown). In addition, there was no difference in the extent of epitope presentation mediated by EtxB-19-mer and EtxB-26-mer conjugates, the first of which lacks the putative cathepsin D cleavage site. As indicated above, only the metalloprotease inhibitor 1,10-phenanthroline was found to inhibit EtxB-induced antigen presentation, suggesting that a metalloprotease may be involved in liberation and/or processing of the EtxB-conjugated peptides. Given the finding that the proteasome can participate in the pathway of EtxB-mediated epitope presentation, it would imply that conjugated peptides are liberated from EtxB and translocated into the cytosol for proteasome processing.

Consequently, it is hypothesized, as depicted in Fig. 8, that EtxB-mediated delivery of peptides into the endogenous class I presentation pathway involves three distinct steps: (i) binding of the EtxB moiety to GM1, which mediates uptake of peptide conjugates into the endosomal pathway; (ii) liberation of the attached peptide, possibly by a metalloprotease; and (iii) translocation of the peptide from an acidic endosome into the cytosolic compartment.

The potential of using the combined targeting and translocating functions of EtxB and the Pol loop segment, respectively, as a generic delivery system for class I epitopes is demonstrated by our results using an epitope derived from influenza virus NP. Here again, the extent of loading of the class I epitope onto MHC-I by the EtxB-20-mer^{NP} conjugate was as efficient as direct loading of surface MHC-I molecules with free 9-mer^{NP} ASNENMETM peptide. The importance of the Pol loop segment in mediating translocation into the class I pathway is highlighted by the finding that the EtxB-10-mer^{NP} conjugate was unable to facilitate loading of MHC-I. The combined abilities, therefore, of EtxB and the Pol-loop segment to efficiently deliver class I-restricted epitopes into the endogenous MHC-I pathway should open up new opportunities for the design of vaccines able to stimulate CTLs. Experiments investigating the potential of such constructs to trigger CTL responses are under way.

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