Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 3530–3534, April 1993
Immunology

Targeted delivery of peptide epitopes to class I major histocompatibility molecules by a modified *Pseudomonas* exotoxin

JOHN J. DONNELLY, JEFFREY B. ULMER, LINDA A. HAWE, ARTHUR FRIEDMAN, XIAO-PING SHI, KAREN R. LEANDER, JOHN W. SHIVER, ALLEN I. OLIFF, DOUGLAS MARTINEZ, DONNA MONTGOMERY, AND MARGARET A. LIU*

Department of Cancer Research, Merck Research Laboratories, West Point, PA 19486

Communicated by Maurice R. Hilleman, January 22, 1993

ABSTRACT **Cytotoxic T lymphocytes (CTLs) expressing the CD8 surface marker recognize peptides in association with major histocompatibility complex (MHC) class I molecules. Although most peptides expressed on MHC class I molecules are derived from self- or virally encoded proteins, delivery of exogenous proteins to the cytosol can result in their being processed for presentation to CTLs on MHC class I molecules. We describe two fusion proteins (PEMa and PENP), consisting of the binding and translocating domains of *Pseudomonas* exotoxin A (PE), fused to peptide epitopes from influenza A matrix protein and nucleoprotein, respectively. These fusion proteins were internalized and processed by MHC class I-positive target cells, resulting in sensitization of target cells for lysis by peptide-specific CTLs. A point mutation known to interfere with intoxication by wild-type PE also reduced the ability of PEMa to sensitize target cells. Fusion of peptide or polypeptide epitopes with PE provides a potential means of eliciting CTLs without the use of self-replicating agents, as well as a useful probe for studying MHC class I-restricted antigen processing.**

Cytotoxic T lymphocytes (CTLs) are an important component of the protective immune response to viral infections (1–4) and tumors (5, 6). Their ability to recognize antigens derived from conserved internal viral proteins (7–10) makes the generation of CTLs capable of providing cross-strain protection a goal of vaccine development. The peptide epitopes recognized by $CD8^+$ major histocompatibility complex (MHC) class I-restricted CTLs are thought to be derived from cytosolic proteins by the action of peptide transporters encoded by the genes TAP.1 and TAP.2 (11, 12). One approach to generating such epitopes for the induction of CTLs has been the delivery of foreign proteins to the cytosol through the use of replicating vectors, such as recombinant bacteria or viruses. However, this method poses risks for the recipient due to the potential pathogenicity of the vectors used (13, 14). A safer alternative is immunization with exogenous proteins; however, such immunization generally results in endocytosis of the antigens, leading to their processing for presentation in association with MHC class II rather than with class I. Thus, a means of delivering exogenous protein antigens into the cytosol under physiologic conditions would potentially be useful for developing CTL vaccines and also would provide a useful probe for the MHC class I-associated-peptide processing pathway (15, 16). Bacterial toxins such as *Pseudomonas aeruginosa* exotoxin A (PE) and *Corynebacterium diphtheriae* toxin are thought to reach the cytosol by means of translocation through endosomal membranes (17, 18) and, therefore, may be used to deliver peptides or proteins into cells (19–21). We describe a modified form of PE in which the binding and translocating domains remain intact but the ADP-ribosylating enzyme (toxin) domain has been replaced with MHC class I-restricted epitopes from influenza A matrix protein or nucleoprotein. These fusion proteins were capable of delivering the epitopes under physiologic conditions to MHC class I molecules and sensitizing target cells for lysis by peptide-specific human and murine CTLs. They provide a unique means of translocating an antigen from the MHC class II to the MHC class I antigen processing pathway.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

MATERIALS AND METHODS

DNA Constructs. The parent plasmid for all PE fusion constructions was pVC45DF+T (22) from I. Pastan (National Institutes of Health, Bethesda, MD). The vector was cut with *Xba* I and *Hin*dIII to remove the OmpA leader sequence. A synthetic linker (5′-CTAGAAATAATTTTGTTTAACTT-TAAGAAGGAGATATACATATGGCCGAAGA-3′) was inserted to rebuild the 5′ end of PE and to put in optimal sequences for expression. The toxin domain was removed by cutting with *Sac* II and *Eco*RI, and all fusions were made by inserting sequences at these sites. *Sac* II cuts the codon for amino acid 413 of PE, so all inserts restore this codon. The vectors pVCPE-X contain the T7 promoter and the codons for amino acids 1–413 of PE, which comprise all of domains Ia, II, and Ib. The insertions for the different vectors were made 3′ to codon 413 of PE. For pVCPEBT, the insert was the sticky-end *Sac* II–*Eco*RI synthetic linker that adds termination codons, 5′-GGCTGATAATAGAGCTCG-3′. For pVCPEMa, the codons for amino acids 57–68 of influenza A matrix protein (M_{57-68}) were generated by PCR amplification from plasmid pAPR701 (23), putting in a *Sac* II site adjacent to codon 57 and a *Sac* I site after codon 68. The fragment was joined as a *Sac* II sticky end and *Sac* I blunt end to the cut vector blunted at the *Eco*RI site. The M_{57-68} sequence is 5′-GGCAACGG-GATTTTAGGATTTGTGTTCACGCTCACCGTGT-GAG-3′. For pVCPENP, a sticky-end *Sac* II–*Eco*RI oligomer linker was synthesized containing the codons for amino acids 147–161 of influenza A nucleoprotein ($NP_{147-161}$), plus the C-terminal 5 amino acids of wild type PE: 5′-GGCACTTAT-CAGAGGACAAGGGCTCTTGTTCGCACCGGAATG-GATCCCCGCGAGGACCTGAAGTG-3′.

The pVCPEMa-276 mutant was made from pVCPEMa by PCR site-directed mutagenesis (24); the PCR primer containing the codon change (underlined) was 5′-CACCCGTCAT-GGCCAGC-3′. The pVCPE-X vectors were grown for expression in *Escherichia coli* BL21(DE3)/pLysE cells as described (25). The glutathione *S*-transferase–M_{57-68} fusion (GST–Ma) was constructed by cutting pGEX-3X (Pharmacia) with *Sma* I and inserting a blunted *Sst* II–*Bsp*EI fragment of

Abbreviations: CTL, cytotoxic T lymphocyte; MHC, major histocompatibility complex; M_{57-68}, amino acids 57–68 of influenza A matrix protein; GST–Ma, glutathione *S*-transferase fused to M_{57-68}; PE, *Pseudomonas aeruginosa* exotoxin A.

*To whom reprint requests should be addressed.

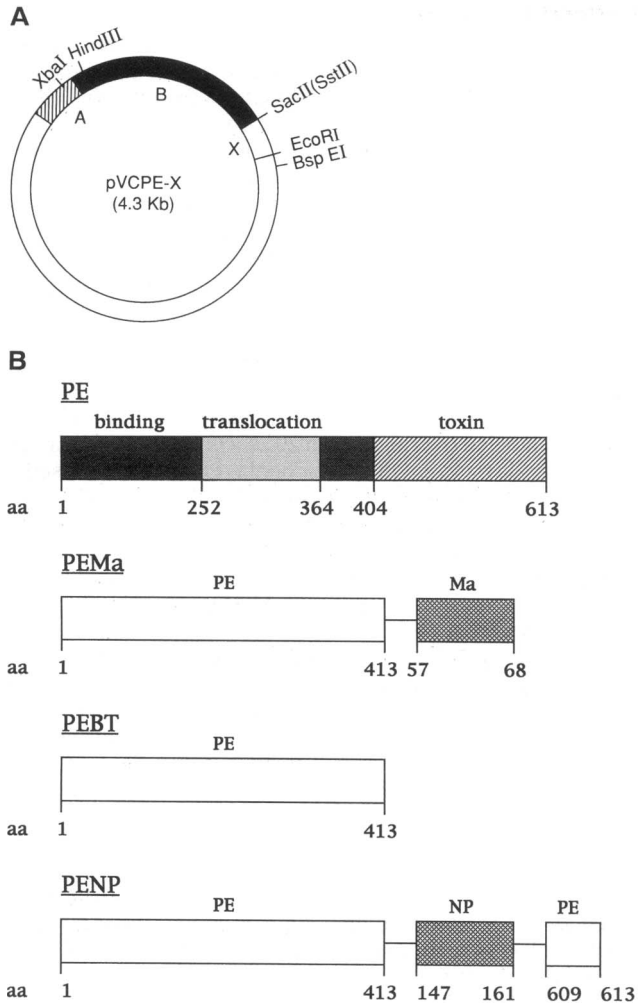

FIG. 1. DNA constructs and fusion proteins. (*A*) DNA constructs for fusion proteins. A, location of T7 promoter; B, coding region for residues 1–413 of PE; X, epitopes (pVCPEMa and pVCPENP) or termination codon (pVCPEBT). (*B*) PE-based fusion proteins. Domains of PE are identified as described by Chaudhary *et al.* (22). Crosshatched areas denote epitope regions derived from matrix protein (Ma) and nucleoprotein (NP) of influenza A. Unshaded areas denote native PE sequence.

the M_{57-68} sequence from pVCPEMa. The GST–Ma fusion protein was purified from *E. coli* as described (26). All synthetic oligonucleotides were purchased from Midland Certified Reagent (Midland, TX).

Purification of Fusion Proteins. Wild-type PE, with binding, translocating, and enzyme domains, and modified PE, with or without peptides containing the influenza A matrix (Lys-Gly-Ile-Leu-Gly-Phe-Val-Phe-Thr-Leu-Thr-Val) or nucleoprotein (Thr-Tyr-Gln-Arg-Thr-Arg-Ala-Leu-Val-Arg-Thr-Gly-Met-Asp-Pro) epitopes, were purified as follows. A sonicated suspension of *E. coli* that had been transfected with plasmid encoding PEMa, PEBT, or PEMa-276 fusion proteins was centrifuged (2800 × *g*, 18°C, 20 min). The supernatant was discarded and the pellet was suspended in 7.5 M urea/100 mM Tris, pH 8.1/100 mM dithiothreitol with aprotinin (0.2 mg/ml), benzamidine (1 mg/ml), and leupeptin (0.2 mg/ml) and extracted for 90 min at 37°C with gentle rocking. The extract was then centrifuged to pellet unextracted material and 30 mg of extracted protein was added to each of two 1500-ml volumes of refolding buffer [100 mM Tris, pH 8.1/25 mM NaCl/10% (vol/vol) glycerol, with protease inhibitors as above] at 4°C. After 98 hr the refolding mixture was filtered through a 0.22-μm-pore-size filter, concentrated on an Amicon YM10 membrane, and chromatographed on a Superdex 75 (Pharmacia) column (2.6 × 60 cm) in Hanks' balanced salt solution (GIBCO) with 2% glycerol, 10 mM Hepes (pH 7.3), penicillin (500 units/ml), and streptomycin (250 μg/ml). *E. coli* BL21(DE3) cells expressing PENP fusion protein were extracted in STET solution [8% sucrose/50 mM Tris, pH 8.1/50 mM EDTA/5% (vol/vol) Triton X-100; protease inhibitors as above were used in all buffers until the chromatography step] for 3 hr at 37°C with shaking, and then sonicated and centrifuged (12,000 × *g*, 30 min). The pellets were washed with 50 mM Tris (pH 8.1), and then solubilized in denaturation solution (6 M guanidine HCl/0.1 M Tris, pH 8.1/0.1 mM EDTA/0.1 M dithiothreitol). PENP (>90% pure by SDS/PAGE) was refolded at 20 μg/ml in 2 M urea/0.1 M Tris, pH 8.1/0.2 mM EDTA/3 mM reduced dithioerythritol/1.25 mM oxidized glutathione at 4°C for 2 days (27). Refolded PENP was concentrated and purified to homogeneity by Mono-Q (Pharmacia) chromatography using a 0–0.5 M NaCl gradient.

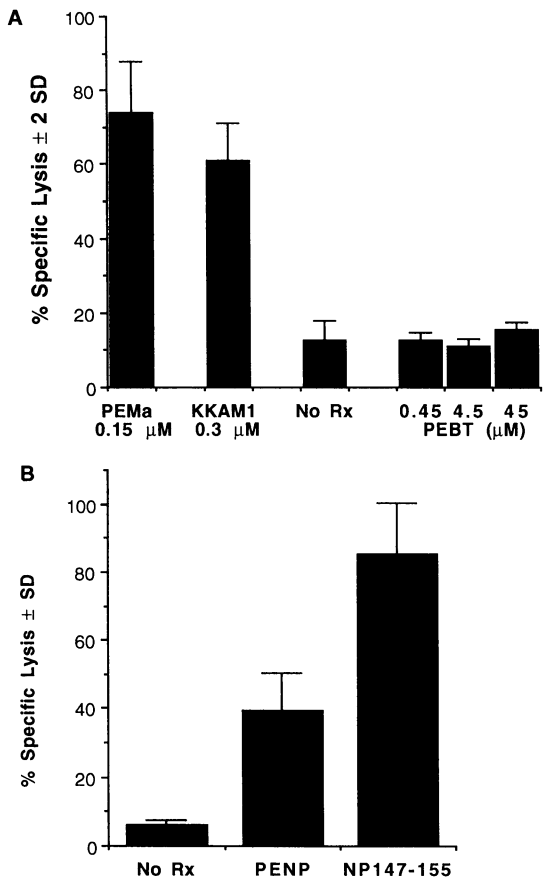

FIG. 2. Specific cytolysis of target cells sensitized with PE fusion proteins by CTLs. (*A*) ^{51}Cr-labeled U-2 OS cell monolayer cultures were treated with the indicated concentrations of PEMa, KKAM1 (a modified form of M_{57-68} with increased solubility, having the sequence Lys-Lys-Ala-Leu-Gly-Phe-Val-Phe-Thr-Leu-Asp-Lys, that has been shown to sensitize HLA-A2.1$^+$ target cells; ref. 28), PEBT, or medium (No Rx) for 30 min at 37°C. The cells were then washed, incubated for a further 2 hr at 37°C, removed from their monolayer cultures, and incubated for 2 hr with CTLs at a 10:1 effector/target (CTL/U-2 OS) ratio. U-2 OS cells were selected for use as targets because they expressed HLA-A2.1 and were sensitive to intoxication by PE at concentrations as low as 5 pM (data not shown). (*B*) P815 cells were incubated with 3 μM PENP, 1 μM $NP_{147-155}$ nucleoprotein peptide, or medium (No Rx) and ^{51}Cr for 2 hr at 37°C, washed, and suspended at 10^5 cells per ml. Nucleoprotein-specific CTLs were plated in triplicate with P815 targets at an effector/target ratio of 30:1 for 3 hr at 37°C.

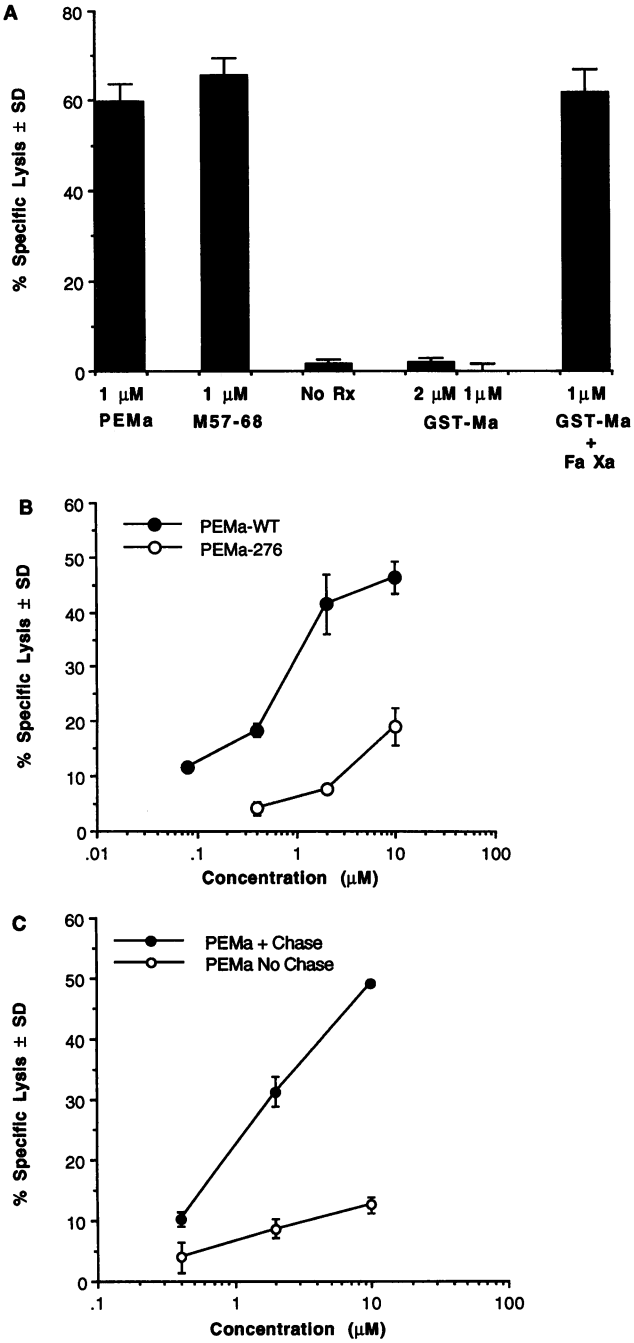

FIG. 3. Processing of PEMa is required for target-cell sensitization. (*A*) ^{51}Cr-labeled U-2 OS cell monolayers were incubated with medium (No Rx) or with PEMa or M_{57-68} at the indicated concentrations for 30 min at 37°C, washed, and incubated at 37°C for a further 4 hr. ^{51}Cr-labeled U-2 OS cell monolayers were incubated with the indicated concentrations of GST–Ma for 4 hr at 37°C. The cleavage of GST–Ma was done with bovine factor Xa (Fa Xa; Boehringer Mannheim) at 25 μg/ml in McCoy's 5A medium supplemented with 15% fetal bovine serum for 4 hr at 37°C. Target cells were then removed from monolayers and plated in triplicate with CTLs at a 20:1 effector/target ratio for 2 hr. (*B*) ^{51}Cr-labeled U-2 OS cell monolayers were incubated with wild-type PEMa (PEMa-WT) or PEMa point mutant at position 276 (PEMa-276), at the indicated concentrations, for 15 min at 37°C. Monolayers were washed, incubated for a further 2 hr at 37°C, and plated in triplicate with CTLs at a 20:1 effector/target ratio for 2 hr. (*C*) ^{51}Cr-labeled HMYA2 cells were incubated with the indicated concentrations of PEMa for 15 min at 4°C, washed with ice-cold RPMI 1640 by centrifugation, and either fixed immediately with ice-cold 0.125% glutaraldehyde (no chase) or incubated a further 3 hr at 37°C and then fixed (chase). HMYA2 cells were selected for this experiment because they are not anchorage-dependent and therefore could be fixed without warming. U-2 OS cells require warming to 37°C for removal from their substrate. HMYA2 cells (28) were maintained in RPMI 1640 with 10% fetal bovine serum and were labeled with 20 μCi of ^{51}Cr for 3 hr at 37°C, exposed to fusion protein or peptide, and resuspended at 10^5 cells per ml. Target cells were plated in triplicate at 0.1 ml per well with 0.1 ml of CTLs at 10^6 per ml, for an effector/target ratio of 10:1, and incubated for 90 min.

Cell Culture. U-2 OS human osteosarcoma cells (American Type Culture Collection) were maintained in McCoy's 5A medium with 15% fetal bovine serum and were removed from flasks with 0.1 M EDTA in phosphate-buffered saline (PBS). P815 mouse mastocytoma cells (American Type Culture Collection) were maintained in Dulbecco's modified Eagle's medium with 15% fetal bovine serum. Human CTLs specific for matrix protein were obtained from a normal HLA-A2.1$^+$ donor and were maintained *in vitro* as described by Bednarek *et al.* (28) with minor modifications. Murine nucleoprotein-specific CTLs were splenic lymphocytes from influenza A nucleoprotein-immunized BALB/c mice that had survived infection with influenza A/HK/68, and were restimulated *in vitro* with autologous spleen cells pulsed with the nucleoprotein epitope peptide 147–155 ($NP_{147-155}$). For CTL assays, U-2 OS cells were plated in 24-well plates at 4×10^5 cells per well and labeled overnight with 20 μCi (740 kBq) of ^{51}Cr. The following day, U-2 OS cell monolayers were exposed to fusion protein or peptide, washed, incubated, and washed with PBS. EDTA (0.1 M in PBS) was then used to detach the cells from the plates, and the cells were washed twice more and suspended at 5×10^4 per ml in RPMI 1640 medium with 10% fetal bovine serum and 0.01 M Hepes. P815 cells were labeled with 20 μCi of ^{51}Cr per 10^6 cells for 3 hr at 37°C, washed by centrifugation, and suspended at 10^5 per ml. Target cells were plated in triplicate at 0.1 ml per well in round-bottomed 96-well plates with 0.1 ml of CTLs at an effector/target ratio of 30:1, 20:1, or 10:1. Assay mixtures were incubated at 37°C and centrifuged, and 20 μl of supernatant was removed for counting in an LKB Betaplate scintillation counter. Results were expressed as percent specific lysis = 100 × [(counts released with CTLs) − (counts released without CTLs)]/[(counts released by 6 M HCl) − (counts released without CTLs)]. Percent error was calculated as 100 × (standard deviations of counts from triplicate wells with CTLs)/[(counts released by 6 M HCl) − (counts released without CTLs)].

Protease Protection. PEMa was labeled with [^{35}S]methionine (1200 Ci/mmol; Amersham) and purified from *E. coli*. ^{125}I-transferrin was purchased from Amersham and used as a positive control. Internalization of PEMa (0.1 μg/ml) was monitored by incubation for 1 hr at 37°C; then the cells were chilled to 4°C and washed. Surface proteins were digested with Pronase (3 mg/ml) for 1 hr at 4°C (29). Cells were sedimented and washed to remove unbound radioactivity. As a measure of adherence of digested PEMa to the cells or incomplete digestion of surface-bound protein, cells were incubated with PEMa at 4°C prior to digestion with Pronase. Radioactivity recovered in the cell pellet under these conditions ranged from 15% to 22% of the total bound.

RESULTS

The expression vectors and fusion proteins used are shown in Fig. 1. A truncated form of PE, consisting of its binding and translocating domains (PEBT), and a hybrid protein containing residues 57–68 of influenza A matrix protein (30) (including the HLA-A2.1-restricted epitope) fused to the truncated PE (PEMa) were expressed in *E. coli*, purified, and refolded. An analogous construct (PENP) containing residues 147–161 of influenza A nucleoprotein (including the K^d-restricted

epitope; ref. 31) was made with an additional five C-terminal amino acids. A mutation of PEMa was expressed with an amino acid change (Arg → Gly) at position 276 (PEMa-276). The corresponding change in PE reduces the activity of the toxin (32, 33) by impairing the ability of PE to be cleaved by a protease in the endosome (32). This mutant is described as a processing mutant and was used to evaluate the requirement of intracellular processing of the fusion protein for the presentation of the MHC class I-restricted epitope.

PEMa efficiently sensitized U-2 OS (HLA-A2.1$^+$) target cells for lysis by M_{57-68}-specific human CTLs, while PEBT, which lacked the M_{57-68} epitope, did not sensitize target cells (Fig. 2*A*). In addition, PENP was capable of sensitizing P815 (H-2^d) target cells for lysis by BALB/c mouse CTLs of appropriate specificity (Fig. 2*B*). Thus, the binding and translocating domains of PE were capable of delivering two different peptide CTL epitopes to an appropriate intracellular compartment for association with MHC class I molecules. In contrast, GST–Ma failed to sensitize even following continuous incubation of GST–Ma with target cells for 4 hr or longer, unless M_{57-68} was specifically released by cleavage of GST–Ma with factor Xa (Fig. 3*A*). Hence, the binding and translocating domains of PE enabled the epitope to be targeted for processing and association with MHC class I molecules. The processing mutant of PEMa sensitized target cells 20- to 50-fold less effectively than did wild-type PEMa (Fig. 3*B*), suggesting that PEMa, like native PE, required delivery to an endosomal compartment and specific proteolysis therein (32).

Internalization of PEMa by target cells was demonstrated by a protease protection approach (29). U-2 OS cells were incubated with [^{35}S]methionine-labeled PEMa at 100 ng/ml for 1 hr at 37°C. After incubation of the cells with labeled PEMa, cells were washed and digested with Pronase (3 mg/ml) for 1 hr at 4°C, and radioactivity was determined for cell pellets and supernatants (29). Approximately 40% of cell-bound PEMa was protected from digestion (5604 cpm bound/14,972 cpm total). The necessity of PEMa to be internalized in order to sensitize target cells for lysis by M_{57-68}-specific CTLs was shown by fixing target cells following exposure to PEMa at 4°C. Cells exposed to PEMa at 4°C and fixed immediately were not sensitized by PEMa. In contrast, cells that were exposed to PEMa at 4°C and then allowed to warm to 37°C prior to fixation were sensitized (Fig. 3*C*). Therefore, when the temperature-sensitive steps of internalization and subsequent processing of PEMa were blocked, PEMa was incapable of sensitizing target cells.

DISCUSSION

The data indicate that the PE fusion proteins acted as vehicles for delivering CTL epitopes into cells, so that the epitopes were able to be recognized by CTLs in association with MHC class I molecules. That the presentation of the epitopes was due to internalization of the fusion proteins and subsequent intracellular processing, rather than to extracellular proteolysis and binding of the epitopes to MHC class I molecules at the cell surface, is indicated by the following lines of evidence: (*i*) the CTL epitope was not cleaved from GST–Ma; (*ii*) the processing mutant, which has an amino acid substitution far removed from the site of the peptide epitope, was 20- to 50-fold less effective at sensitizing target cells for lysis; (*iii*) improperly folded PEMa (urea-extracted, not refolded, shown to not bind to the PE receptor) did not sensitize target cells (data not shown); and (*iv*) PENP sensitized target cells using a CTL epitope that is internal within the fusion protein and, therefore, may require two or more separate and specific cleavage events.

The point mutation introduced into PEMa allows processing steps common to PEMa and PE to be identified. Cleavage of PE at residue 279 is an obligatory step during PE processing and occurs after it is internalized to an endosomal compartment (32, 33). The Arg^{276} → Gly mutation increases the resistance of PE to this cleavage and thereby reduces its toxicity (32). The ability of this processing mutation to reduce sensitization by PEMa indicates that this specific proteolysis, most likely in an endosomal compartment, also is required for sensitization. Hence, PEMa and PE appear to share similar processing and trafficking pathways.

We have shown that exogenously applied PEMa and PENP are capable of delivering their respective epitopes to MHC class I molecules, resulting in sensitization of target cells for lysis by CTLs. Sensitization occurs most effectively when the epitope is part of a properly folded fusion protein consisting of intact processing and translocating regions derived from the toxin. Although peptides (which were larger than the minimal epitope) were utilized, entire proteins also could be inserted into the fusion proteins, an advantage for a potential vaccine. Thus, an exogenous protein which normally enters the MHC class II processing pathway can be targeted to a cellular processing pathway, resulting in presentation of a viral epitope to CTLs by MHC class I molecules. This occurs under physiologic conditions, in contrast to other techniques which have been used to deliver antigens for processing and presentation on MHC class I molecules (34, 35). This observation has implications for the development of vaccines designed to elicit CTLs without the use of self-replicating agents. Furthermore, these fusion proteins are useful probes for studying MHC class I-restricted antigen processing and the intersection between endocytic and exocytic pathways.

J.J.D. and J.B.U. contributed equally to this work. We thank R. R. Deck, H. Perry, C. Shi, and J. Shieh for technical assistance; M. Gammon and J. Hermes for reagents and helpful discussions; and M. R. Hilleman, A. R. Williamson, and H. Zweerink for critical review of the manuscript.

1. Rouse, B. T., Norley, S. & Martin, S. (1988) *Rev. Infect. Dis.* **10**, 16–33.
2. Lukacher, A. E., Braciale, V. L. & Braciale, T. J. (1984) *J. Exp. Med.* **160**, 814–826.
3. McMichael, A. J., Gotch, F. M., Noble, G. R. & Beare, P. A. S. (1983) *N. Engl. J. Med.* **309**, 13–17.
4. Wraith, D. C., Vessey, A. E. & Askonas, B. A. (1987) *J. Gen. Virol.* **68**, 433–440.
5. Cerundolo, V., Lahaye, T., Horvath, C., Zanovello, P., Collavo, D. & Engers, H. E. (1987) *Eur. J. Immunol.* **17**, 173–178.
6. Kast, W. M., Offringa, R., Peters, P. J., Voordouw, A. C., Meloen, R. H., van der Eb, A. J. & Melief, C. J. M. (1989) *Cell* **59**, 603–614.
7. Bjorkman, P. J., Saper, M. A., Samraoui, B., Bennett, W. S., Strominger, J. L. & Wiley, D. C. (1987) *Nature (London)* **329**, 512–518.
8. Madden, D. R., Gorga, J. C., Strominger, J. L. & Wiley, D. C. (1991) *Nature (London)* **353**, 321–325.
9. Townsend, A. R. (1989) *Annu. Rev. Immunol.* **7**, 601–24.
10. Yewdell, J. W., Eisenlohr, L. C., Cox, J. H., Russ, G. & Bennink, J. R. (1990) in *The Year in Immunology*, eds. Cruse, J. M. & Lewis, R. E. (Karger, Basel), pp. 38–50.
11. Arnold, D., Driscoll, J., Androlewicz, M., Hughes, E., Creswell, P. & Spies, T. (1992) *Nature (London)* **360**, 171–173.
12. Momburg, F., Ortiz-Navarrete, V., Neefjes, J., Goulmy, E., van de Wal, Y., Spits, H., Powis, S. J., Butcher, G. W., Howard, J. C., Walden, P. & Hammerling, G. J. (1992) *Nature (London)* **360**, 174–176.
13. Redfield, R. R., Wright, D. C., James, W. D., Jones, T. S., Brown, C. & Burke, D. S. (1987) *N. Engl. J. Med.* **316**, 673–676.
14. Mascola, L., Sorvillo, F., Neal, J., Iwakoshi, K. & Weaver, R. (1989) *Arch. Intern. Med.* **149**, 1569–1572.
15. Yewdell, J. W., Bennink, J. R. & Hosaka, Y. (1988) *Science* **239**, 637–640.
16. Riddell, S. R., Rabin, M., Geballe, A. P., Britt, W. J. & Greenberg, P. D. (1991) *J. Immunol.* **146**, 2795–2804.

17. Sandvig, K. & Olsnes, S. (1982) *J. Biol. Chem.* **257,** 7504–7513.
18. Marnell, M. H., Shia, S.-P., Stookey, M. & Draper, R. K. (1984) *Infect. Immun.* **44,** 145–150.
19. Yewdell, J. W. & Bennink, J. R. (1990) *Cell* **62,** 203–206.
20. Prior, T. I., FitzGerald, D. J. & Pastan, I. (1991) *Cell* **64,** 1017–1023.
21. Stenmark, H., Moskaug, J. O., Madshus, I. H., Sandvig, K. & Olsnes, S. (1991) *J. Cell Biol.* **113,** 1025–1032.
22. Chaudhary, V. K., Xu, Y.-H., FitzGerald, D., Adhya, S. & Pastan, I. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 2939–2943.
23. Young, J. F., Desselberger, U., Graves, P., Palese, P., Shatzman, A. & Rosenberg, M. (1983) in *The Origin of Pandemic Influenza Viruses*, ed. Laver, W. G. (Elsevier, Amsterdam), pp. 129–138.
24. Higuchi, R. (1990) in *PCR Protocols: A Guide to Methods and Applications*, eds. Innis, M. A., Gelfand, D. H., Sminsky, J. J. & White, T. J. (Academic, San Diego), pp. 177–183.
25. Studier, F. W., Rosenberg, A. H., Dunn, J. J. & Dubendorff, J. W. (1990) *Methods Enzymol.* **185,** 60–89.
26. Smith, D. B. & Johnson, K. S. (1988) *Gene* **67,** 31–40.
27. Buchner, J. & Rudolph, R. (1991) *Bio/Technology* **9,** 157–162.
28. Bednarek, M. A., Engl, S. A., Gammon, M. C., Linquist, J. A., Porter, G., Williamson, A. R. & Zweerink, H. J. (1991) *J. Immunol. Methods* **139,** 41–47.
29. Sandvig, K., Olsnes, S., Petersen, O. W. & van Deurs, B. (1987) *J. Cell Biol.* **105,** 679–689.
30. Gotch, F., Rothbard, J., Howland, K., Townsend, A. & McMichael, A. (1987) *Nature (London)* **326,** 881–882.
31. Townsend, A. R., Gotch, F. M. & Davey, J. (1985) *Cell* **42,** 457–467.
32. Jinno, Y., Ogata, M., Chaudhary, V. K., Willingham, M. C., Adhya, S., FitzGerald, D. J. & Pastan, I. (1989) *J. Biol. Chem.* **264,** 15953–15959.
33. Ogata, M., Chaudhary, V. K., Pastan, I. & FitzGerald, D. J. (1990) *J. Biol. Chem.* **265,** 20678–20685.
34. Moore, M. W., Carbone, F. R. & Bevan, M. J. (1988) *Cell* **54,** 777–785.
35. Harding, C. V. (1992) *Eur. J. Immunol.* **22,** 1865–1869.