INFECTION AND IMMUNITY, Oct. 1995, p. 3851–3857 Vol. 63, No. 10
0019-9567/95/$04.00+0

Cell-Invasive Activity of Epitope-Tagged Adenylate Cyclase of *Bordetella pertussis* Allows In Vitro Presentation of a Foreign Epitope to CD8$^+$ Cytotoxic T Cells

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Received 11 July 1995/Accepted 27 July 1995

The adenylate cyclase (AC) toxin (CyaA) of *Bordetella pertussis* has an invasive catalytic domain (AC domain) which penetrates the cytoplasmic membrane of a variety of eukaryotic cells and intoxicates them by unregulated synthesis of cyclic AMP. Previous work led to identification of five permissive sites in the AC domain at which heterologous peptides are accommodated without affecting its enzymatic properties. We have constructed a set of CyaA toxins tagged at these permissive sites by insertion of a CD8$^+$ T-cell epitope, RPQASGVYMGNLTAQ, from the nucleoprotein of lymphocytic choriomeningitis virus. Introduction of the epitope at any of the five sites did not affect the capacity of the toxin to deliver its AC domain into target cells. Moreover, the toxin with the inserted epitope was shown to sensitize target cells for lysis by epitope-specific CD8$^+$ cytotoxic T lymphocytes in vitro, showing that the tagged AC was processed for presentation of the lymphocytic choriomeningitis virus epitope in association with the major histocompatibility complex class I molecules. This finding indicates that by virtue of delivery of foreign epitopes into the antigen-presenting cells, purpose-designed recombinant CyaAs may be useful for induction of specific major histocompatibility complex class I-restricted cell-mediated immunity also in vivo.

The adenylate cyclase (AC) toxin (ACT, CyaA) is an important virulence factor of *Bordetella pertussis*, the causative agent of whooping cough (17, 22, 35). It is expressed from its structural gene, *cyaA*, which encodes a 1,706-residue-long protein with two different activities. The toxin has a calmodulin (CaM)-activated AC activity located in the N-terminal 400 residues and hemolytic activity, corresponding to the C-terminal 1,300 residues of the molecule (15, 16). Both activities can be expressed independently as stable and active truncated proteins (14, 29). ACT is synthesized as an inactive precursor and is converted to the active toxin by palmitoylation of the ε-amino group of Lys-983 in the presence of the accessory protein CyaC (2, 19, 21). The toxin is then secreted by a dedicated secretion apparatus involving the products of *cyaBDE* (16). The hemolytic moiety of ACT harbors four channel-forming hydrophobic segments, 38 copies of the characteristic glycine and aspartic acid-rich nonapeptide repeats, and a C-terminal secretion signal (4, 15, 33).

The cytotoxic activity of ACT is due to its capacity to penetrate a variety of eukaryotic cells and to produce high and unregulated levels of cyclic AMP (cAMP), which impairs their physiological functions. This activity is the major contribution of ACT to the pathogenesis of pertussis (7, 18, 22). The steps involved in the cytotoxic activity, i.e., the insertion of the toxin into membranes and the penetration of AC into cells, as well as the mechanism of intracellular AC inactivation, are poorly understood.

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We have recently identified within the truncated catalytic domain of AC several permissive sites which tolerate amino acid insertion without interfering with the enzymatic activity (23). We reasoned that by inserting foreign antigenic determinants into defined permissive sites within the full-length toxin, the resulting epitope-tagged ACTs might become a useful tool for analysis of the different steps involved in toxin action. Furthermore, because this toxin penetrates directly into the cytoplasmic compartment of target cells, it may upon processing yield free epitope peptides, as if they were synthesized inside the cells. We therefore used a CD8$^+$ T-cell epitope of lymphocytic choriomeningitis virus (LCMV) for insertion into the recombinant toxins (1, 36). We show here that insertion of the epitope at any of the five permissive sites did not affect the capacity of the toxin to deliver the tagged AC domain into target cells. Moreover, we demonstrate that such a tagged toxin can sensitize target cells for killing by peptide-specific CD8$^+$ cytotoxic T lymphocytes (CTLs) in vitro, suggesting that the foreign epitope was delivered into the major histocompatibility complex (MHC) class I-restricted antigen processing pathway.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth media. *Escherichia coli* XL1-Blue (Stratagene) was used throughout this work for DNA manipulation and for expression of ACs and CyaAs. The AC-deficient *E. coli* strain TP610 (20) was used for functional assays of the modified ACs by in vivo complementation. Plasmids pACM, pCACT3, and pLG575 have been described elsewhere (5, 23, 25). Transformants were selected on MacConkey or LB agar medium containing 100 mg of ampicillin per liter. The LB-blood-agar plates contained 5% defibrinated sheep blood.

Insertion of epitopes into AC and CyaA. The in vitro DNA manipulations were performed according to standard protocols (30). Construction of plasmids pACM-LCMV and pCACT-LCMV for expression of the AC enzymes and the toxins bearing the epitopes is described in Fig. 1. The epitope-encoding double-stranded synthetic oligonucleotide 5′-dCGT CCG CAA GCT TCT GGT GTT TAC ATG GGT AAC CTG ACC GCT CAG G-3′ was inserted between codons

235 and 236 of *cyaA*, and 5′-dT CGT CCG CAA GCT TCT GGT GTT TAC ATG GGT AAC CTG ACC GCT CAG-3′ was inserted at the other positions (only sequences of coding strands are given). The oligonucleotides contain a unique *Hin*dIII restriction site (underlined).

Purification of toxins. The bacterial cultures and extraction of toxins with 8 M urea were performed as previously described (32). The toxins were purified by chromatography on DEAE-Sepharose under denaturing conditions. The extracts supplemented with 0.15 M NaCl were applied to columns (1.5 volume of packed gel per volume of urea extract) equilibrated with 8 M urea–0.15 M NaCl in 50 mM Tris-HCl (pH 8.0), and the columns were washed with 2 bed volumes of the equilibration solution. The adsorbed 200-kDa CyaAs were eluted with 8 M urea–2 M NaCl in 50 mM Tris-HCl (pH 8.0). The fractions containing toxins were pooled, concentrated by ultrafiltration on an Amicon YM-10 membrane, and further purified by affinity chromatography on CaM-agarose as previously described (32) and eluted with 8 M urea–50 mM Tris-HCl (pH 8.0)–2 mM EDTA.

Assay of AC, invasive AC, and hemolytic activities. AC activity was measured as previously described (24) in medium containing 1 μM bovine brain CaM and 2 mM [α-^{32}P]ATP (1×10^5 to 2×10^5 cpm per assay). One unit of AC activity corresponds to 1 μmol of cAMP formed in 1 min at 30°C and pH 8.

Internalized (invasive) AC and hemolytic activities of CyaAs were determined on toxin-treated sheep erythrocytes (5×10^8/ml) essentially as previously described (3). Toxin samples of the different CyaA-derived proteins were adjusted to 40 U/ml in 50 mM Tris-HCl (pH 8.0)–8 M urea–2 mM EDTA, and 35 μl of each toxin was directly diluted (100-fold) into 3.5 ml of prewarmed suspensions of sheep erythrocytes (5×10^8/ml) in TNC (10 mM Tris-HCl [pH 8.0], 150 mM NaCl, 2 mM $CaCl_2$). The amount of AC activity which had penetrated into the erythrocytes was determined after 30 min of incubation at 37°C. Aliquots of the suspensions were chilled on ice and washed; tosylsulfonyl phenyalanyl chloromethyl ketone (TPCK)-trypsin was added, and the suspensions were incubated for 10 min at 37°C to hydrolyze the AC remaining outside the erythrocytes. After addition of soybean trypsin inhibitor (twofold excess), the erythrocytes were washed and lysed with 0.1% Triton X-100. The internalized AC activity protected from trypsin digestion was then measured. The hemolytic activity was determined by measuring the percentage of erythrocyte lysis as a function of time. The erythrocyte suspensions were centrifuged 5 min at $1{,}000 \times g$. The percentage of lysis was calculated from the amount of hemoglobin (measured by the A_{541}) released into the supernatant, taking as 100% the A_{541} obtained upon detergent-induced lysis.

Preparation of murine anti-p118-132 sera. The conjugate for mouse immunization was prepared by coupling of the synthetic peptide p(118-132) (RPQASG VYMGNLTAQ), corresponding to the *H-2*^{d} T-cell epitope of the LCMV nucleoprotein, to ovalbumin (34). Two milliliters of a peptide solution at 3 mg/ml was mixed with 4 ml of an ovalbumin solution (1 mg/ml in phosphate-buffered saline [PBS]), and 6 ml of 2% glutaraldehyde solution (Sigma) was added slowly. After a 2-h incubation at room temperature, the conjugate was dialyzed against PBS for 48 h at 4°C and stored frozen. Each of 10 BALB/c female mice (6 to 10 weeks old) was immunized subcutaneously with 20 μg of this p(118-132)/ovalbumin conjugate emulsified in complete Freund adjuvant (Sigma). On days 21 and 42, the mice received boost injections of 20 μg of conjugate in incomplete Freund adjuvant (Sigma). The mice were bled 10 days after the last injection, and the obtained sera were pooled.

Generation of CTL responses. On days 0 and 7, BALB/c mice were immunized subcutaneously at the base of the tail with 100 μg of p(118-126) in incomplete Freund adjuvant as previously described (10, 36). On days −1 and 0, mice received intraperitoneal injections of 0.5 mg of anti-CD4 monoclonal antibody GK 1-5 prepared as previously described (10), since we recently showed that this treatment increased p(118-126)-induced CTL responses (unpublished observations). One week after the last injection of peptide, the animals were killed and the spleens were removed aseptically. A single-cell suspension of spleen cells was prepared in RPMI 1640 medium (Seromed, Berlin, Germany) containing 2 mM L-glutamine, antibiotics, 5×10^{-5} M 2-mercaptoethanol and 10% fetal calf serum. Mixed lymphocyte cultures (MLC) were established with 2.5×10^7 responding cells and 2.5×10^7 irradiated (2,500 rad) stimulator syngeneic spleen cells in 10 ml of medium containing 0.05 μM p(118-126) peptide. The cultures were seeded at 37°C into upright T25 flasks for 5 days.

Cytotoxicity assay. Lymphocytes harvested from MLC were tested for cytotoxicity in a 6-h ^{51}Cr release assay in 96-well flat bottom plates (Nunc) on 10,000 ^{51}Cr-labeled BALB/3T3 (*H-2*^{d}) target cells (27). In some experiments, cultured effector cells were depleted of CD8$^+$ T cells by incubation with anti-CD8 monoclonal antibody H35.17.2 prepared as previously described (10) and anti-rat immunoglobulin-coated magnetic beads (Dynabeads; Dynal A.S., Oslo, Norway).

Target cells were labeled overnight with ^{51}Cr in microplates and washed three times with balanced salt solution (10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES; pH 7.4], 140 mM NaCl, 5 mM KCl, 3 mM $MgCl_2$, 2 mM $CaCl_2$, and 1% glucose). Effector cells were then added to target cells at various effector/target ratios in balanced salt solution together with 10 μg of either the p(118-126) peptide or the different toxins per ml.

After 6 h, the plates were centrifuged, the supernatants were removed, and the released radioactivity was measured with a gamma counter. Spontaneous release and maximal release of radioactivity were determined by incubating the target cells (six wells) in medium alone or with 1 N HCl. Spontaneous release was less than 20% of the maximal release. The percentage of specific ^{51}Cr release was calculated as [(experimental release − spontaneous release)/(maximal release − spontaneous release)] × 100. Results were expressed as the mean ± standard error of two wells per group and were compared by the Student *t* test. $P > 0.05$ was considered nonsignificant.

Materials and experimental animals. Oligonucleotides were synthesized by the Organic Chemistry Unit, Institut Pasteur (Paris, France). The peptides RPQASGVYMGNLTAQ [p(118-132)] and RPQASGVYM [p(118-126)] were purchased from Neosystem (Strasbourg, France). The sheep blood for cytotoxicity assays was always taken from the same animal (animal 322) at the Institute Pasteur Farm (Rennemoulin, France). BALB/c female mice, 6 to 10 weeks old, were bred in the animal facilities of the Institut Pasteur.

Standard techniques. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis, electrotransfer of proteins to nitrocellulose membranes, and immunoblotting were performed according to standard protocols (30). Protein concentrations were determined by using a Micro BCA protein assay reagent kit (Pierce).

RESULTS

Construction of epitope-tagged CyaA toxins. In a first attempt to generate tools for studies of translocation of the AC domain of CyaA across membranes, we constructed a set of five toxins tagged with a 15-residue-long T-cell epitope (RPQASGVYMGNLTAQ) of the LCMV nucleoprotein (1). It was previously shown that heterologous peptides can be accommodated at five permissive sites of the AC domain of CyaA without affecting its enzymatic properties (23). Therefore, the oligonucleotides encoding the LCMV epitope were inserted into the truncated *cyaA* gene, encoding the AC domain, using the unique *Pst*I sites placed at the five permissive positions between codons 137 and 138, 224 and 225, 228 and 229, 235 and 236, and 317 and 318 (23) on the pACM constructs (Fig. 1).

Two direct phenotypic screening procedures were used for identification of constructs with the desired inserts. The first screening was based on complementation of *E. coli* TP610, which lacks the endogenous AC and yields white colonies on MacConkey-maltose indicator plates (20). This strain was complemented by constructs producing an active AC domain of CyaA (23). For this purpose, an in-frame stop codon was introduced into the complementary strand of the oligonucleotide encoding the LCMV epitope. Consequently, upon insertion of the oligonucleotide in the incorrect orientation or upon a frameshift, introducing self-ligation of the acceptor plasmids without insert, only inactive truncated ACs could be produced. Hence, only the desired constructs with the oligonucleotide inserted in the right orientation could yield functional ACs and could correct the defect in cAMP synthesis of strain TP610. Such clones could be directly identified as red colonies on the indicator plates. The orientations and sequences of the oligonucleotide inserts of all of the pACM-LCMV constructs were verified by local DNA sequencing.

The fragments encoding the epitope-tagged catalytic domains on the pACM-LCMV plasmids were used to replace the corresponding portion of the wild-type *cyaA* gene on pCACT3 (Fig. 1). This plasmid bears both the full-length *cyaA* gene and the gene for the toxin-activating protein CyaC and allows overproduction of the active toxin in *E. coli*. The clones producing the desired toxin constructs, with the tagged AC domains correctly fused to the hemolysin part of CyaA, were directly identified as hemolytic transformants of the secretory strain XL1-Blue(pLG575) on blood agar plates (33). The physical maps of the corresponding pCACT-LCMV plasmids were controlled by restriction analysis, and the produced toxins were further characterized biochemically.

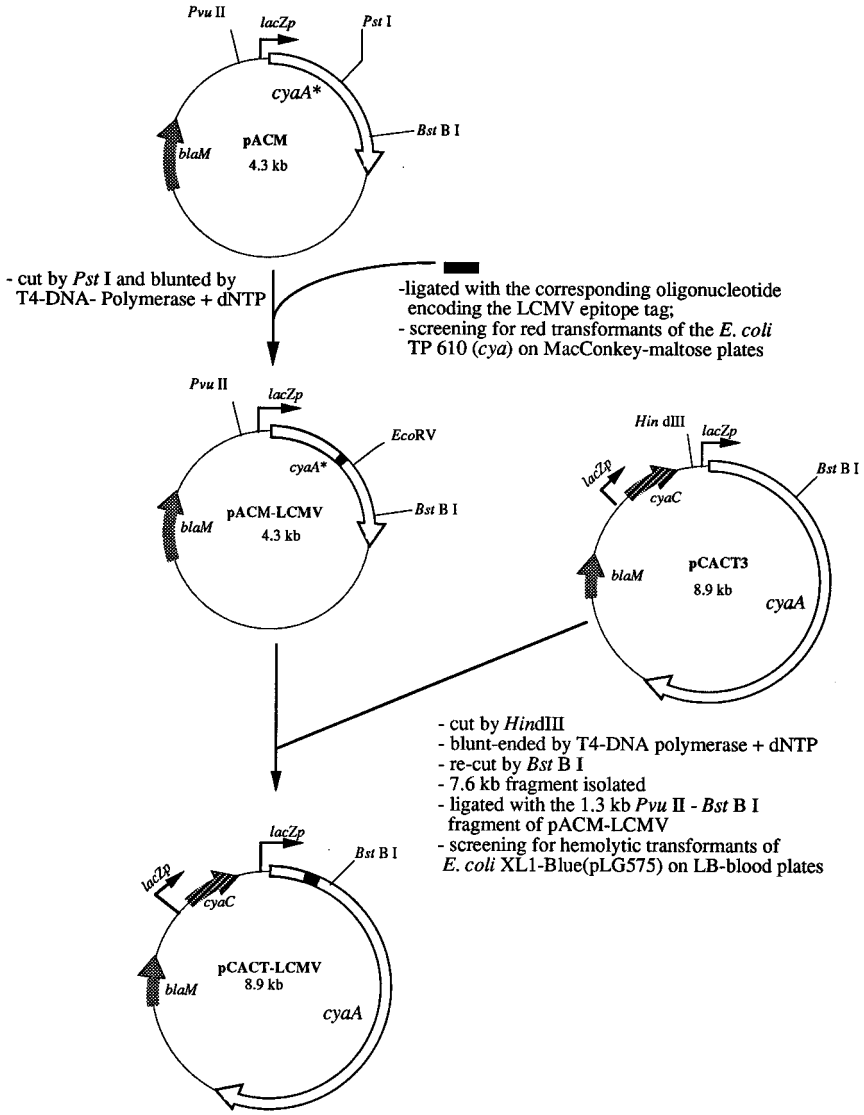


FIG. 1. Schematic diagram of the procedure used for construction of toxins bearing the LCMV epitope. Synthetic oligonucleotides encoding the LCMV epitope were introduced into the pACM plasmids at the permissive sites between codons 137 and 138, 224 and 225, 228 and 229, 235 and 236, and 317 and 318 of *cyaA* (23). The correct insertion of the oligonucleotides in the resulting pACM-LCMV plasmids was verified by local DNA sequencing. In the second step, the 5′ portions of the *cyaA* gene with the inserted oligonucleotide were used to replace the wild-type sequence in the *cyaA* gene on pCACT3. dNTP, deoxynucleoside triphosphate.

Insertion of the epitope does not substantially affect enzymatic properties and cytotoxic activity of CyaA. It was essential to characterize the enzymatic properties of the full-length CyaA-LCMV proteins prior to examination of their toxic properties. For this purpose, we developed a rapid purification protocol employing DEAE-Sepharose chromatography in the presence of 8 M urea followed by CaM-agarose chromatography (see Materials and Methods). This new purification scheme reduced the proteolytic breakdown of the cytotoxic 200-kDa form of CyaA upon dilution of the urea extracts and yielded better separation of the full-length CyaA from its enzymatically active, proteolytic fragments that copurify with the toxin on the conventional single step CaM-agarose columns. As shown by SDS-PAGE analysis in Fig. 2A, the CyaA-LCMV proteins could be purified from urea extracts close to homogeneity. When probed with specific polyclonal murine sera raised against the inserted LCMV epitope, only the tagged toxins, not the wild-type CyaA, were specifically recognized in Western blots (immunoblots) (Fig. 2B).

The specific AC activities of the tagged toxins at 100 μM CaM were similar to that of the wild-type CyaA (Table 1), with the exception of the construct CyaA137LCMV, which reproducibly required higher CaM concentrations for activation. The apparent K_{act} values for CaM activation of all other purified constructs were close to that of the wild type. These data show that the enzymatic properties of recombinant ACTs were not affected by insertion of the epitope tags at the permissive sites of their AC domains, thereby confirming the versatility of the permissive sites in the full-length CyaA, previously observed with the isolated AC domain bearing random inserts.

In contrast to the various 45-kDa ACs corresponding to the catalytic domain of the CyaAs, which exhibit sharp CaM activation curves with half-maximal activation in the range of 0.2 to 1 nM CaM, the CaM activation curves for the full-length

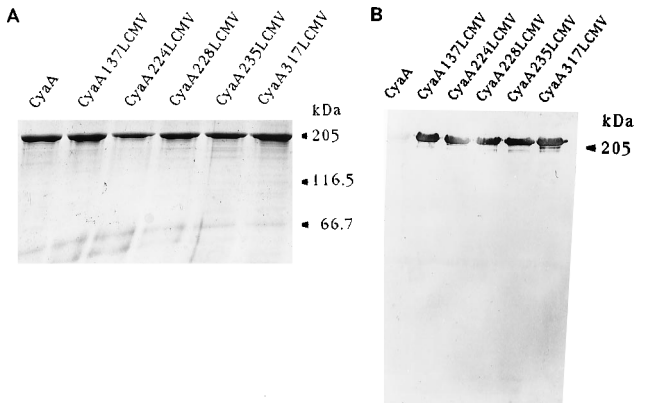
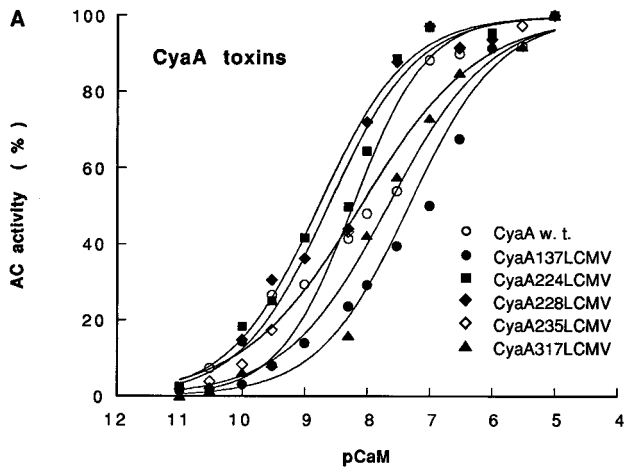


FIG. 2. Analysis of the purified constructs. (A) SDS-PAGE analysis of the purified CyaA-LCMV proteins. Five-microgram aliquots of the purified proteins were separated on a 7.5% acrylamide gel and visualized by Coomassie blue staining. (B) Immunoblot of the epitope-tagged proteins. The proteins were separated by SDS-PAGE and electrotransferred to a nitrocellulose membrane. After blocking of the membrane and incubation with a specific polyclonal murine serum raised against the LCMV epitope, the specifically recognized proteins were decorated by a second antibody coupled to alkaline phosphatase.



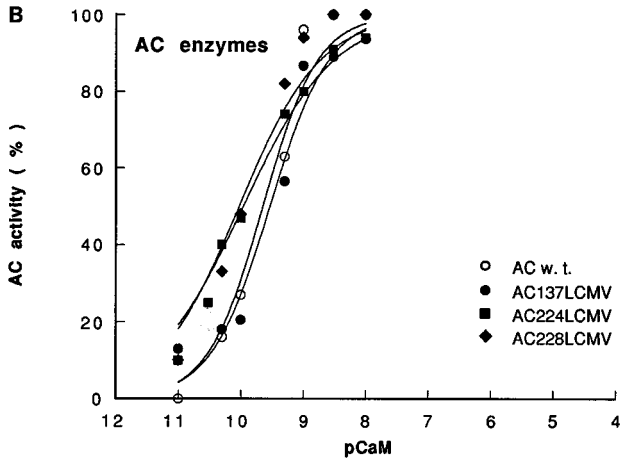


FIG. 3. Activation of the tagged ACs and CyaA toxins by CaM. The AC activities of fixed amounts of the CyaA toxins (A) and AC enzymes (B) were determined in the presence of various concentrations of bovine brain CaM as previously described (23). The measured AC activities are expressed as percentages of the activity obtained in the presence of 100 μM CaM (100%). w.t., wild type.

form of CyaA and its CyaA-LCMV derivatives were extended (Fig. 3).

The cytotoxic and hemolytic activities of the CyaA-LCMV constructs were determined by using sheep erythrocytes as model target cells (Fig. 4). Equivalent amounts of the purified proteins were added to washed erythrocytes, and their capacity to deliver the tagged AC moieties into the cells was measured as the accumulation of internalized AC activity (Fig. 4A). No protection of AC against externally added trypsin (Fig. 4A) could be observed with the noncytotoxic proCyaA protein, purified from a strain lacking the toxin-activating protein CyaC, which served as a control for the specificity of the assay. All tagged toxins preserved their cell-invasive AC activity, and no significant difference from the activity of the wild-type CyaA was found. In parallel, the kinetics of intracellular cAMP production in the erythrocytes treated by the different proteins was measured (not shown), and the results were the same as for the assay of internalized AC activity. It can therefore be concluded that not only the enzymatic activity but also the full cytotoxic (invasive AC) activity was preserved in the epitope-tagged CyaA-LCMV proteins. In addition, the hemolytic activity of the tagged toxins was not significantly altered by insertion of the epitopes into the catalytic domain of CyaA (Fig. 4B).

The LCMV CTL epitope inserted into ACT is presented to CD8$^+$ CTLs. Since the insertion of the LCMV epitope did not affect the capacity of the toxin to deliver the tagged AC domain into target cells, we chose one of these hybrid molecules for analysis of its capacity to deliver the foreign epitope into the MHC class I-restricted antigen processing pathway in vitro.

Immunization with the free p(118-132) peptide in incomplete Freund adjuvant was previously shown to induce CD8$^+$ T-cell-mediated, class I-restricted CTL responses (1, 10). How-

TABLE 1. Characteristics of CyaA variants bearing the epitope insertions

Toxin	Inserted sequence[a]	AC sp act (U/mg of protein)	K_{act} for CaM (nM)[b]
Wild-type CyaA	None	403 ± 28	7–17
CyaA137LCMV	V_{137}**ARPQASGVYMGNLTAQ**A_{138}	383 ± 21	70–109
CyaA224LCMV	R_{224}**ARPQASGVYMGNLTAQ**A_{225}	412 ± 23	3–12
CyaA228LCMV	E_{228}**ARPQASGVYMGNLTAQ**A_{229}	391 ± 27	4–11
CyaA235LCMV	R_{235}**RPQASGVYMGNLTAQG**E_{236}	385 ± 33	6–15
CyaA317LCMV	S_{317}**ARPQASGVYMGNLTAQ**A_{318}	405 ± 19	12–19

[a] Boldface characters indicate inserted amino acids (one-letter code) at defined positions of wild-type CyaA; the sequence of the epitope is underlined.
[b] Determined from curves of AC activity as function of CaM concentration. Ranges were determined for each protein in five independent experiments.

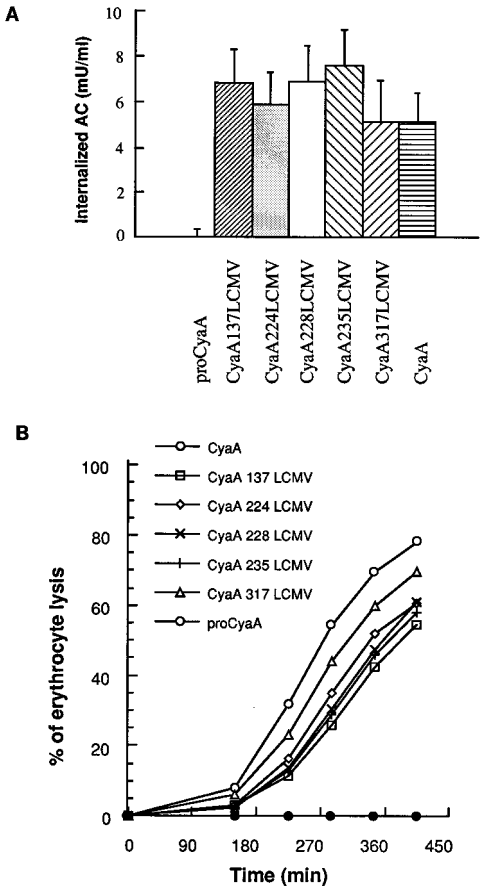


FIG. 4. Cytotoxic and hemolytic activities of the tagged toxins. The cytotoxic (internalized AC) (A) and hemolytic (B) activities of the wild-type and tagged CyaAs were determined on sheep erythrocytes as described in Materials and Methods. The average of data obtained in three different experiments using two different batches of toxin preparations is shown. The bars indicate standard errors.

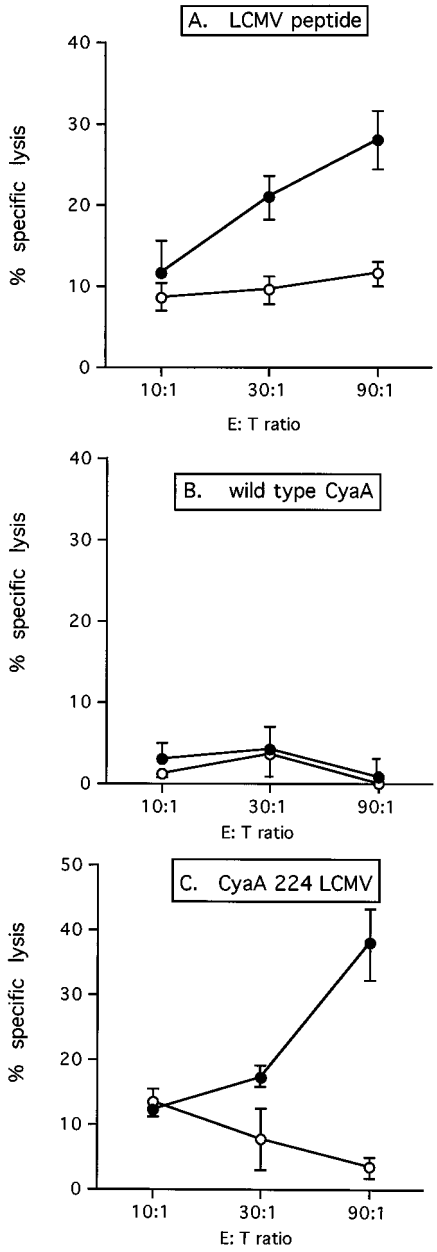


FIG. 5. $CD8^+$ T cells kill target cells incubated with the recombinant CyaA toxin bearing the LCMV T-cell epitope. Polyclonal CTLs specific for the nucleoprotein LCMV $CD8^+$ T-cell epitope were induced in BALB/c mice by subcutaneous injection of 100 μg of p(118-126) in incomplete Freund adjuvant. Seven days after immunization, mouse spleen cells were in vitro stimulated with p(118-126) peptide. After 5 days, total (●) or $CD8^+$-depleted (○) cultured cells were incubated for 6 h at different effector/target (E:T) ratios with ^{51}Cr-labeled BALB/3T3 target cells in the presence of 10 μg of p(118-126) peptide (A), wild-type CyaA (B), or CyaA224LCMV (C) per ml. Results are expressed as mean percentage of ^{51}Cr release ± standard error of two wells per group.

ever, more recently, the optimal 9-mer p(118-126) was shown to elicit stronger antipeptide CTL responses in vivo (36). This peptide was therefore used in the following experiments. Polyclonal anti-LCMV CTLs were induced by two injections of 100 μg of the p(118-126) peptide in BALB/c mice, and then spleen cells were stimulated with the peptide in vitro. It was previously demonstrated that CTL responses induced under these conditions are mediated by $CD8^+$ T cells and are MHC class I restricted (1, 10). The capacity of these effector cells to lyse labeled target cells incubated either with the peptide or with the hybrid toxin was then analyzed. A minimal lysis was observed when effector cells were incubated for 6 h with target cells either with medium alone (data not shown) or in the presence of the wild-type ACT toxin lacking the LCMV epitope at a concentration of 10 μg/ml (Fig. 5B). In contrast, a specific lysis was observed when the target cells were incubated with the free peptide (Fig. 5A) or the CyaA224LCMV toxin bearing the LCMV epitope, each at 10 μg/ml (Fig. 5C). At effector/target ratios of 30:1 and 90:1, a significant difference ($P < 0.05$) was observed between the lysis of untreated target cells and the lysis of target cells incubated with the recombinant CyaA224LCMV. A specific lysis was also observed after incubation of the target cells with 1 μg of CyaA224LCMV per ml, whereas incubation with 30 μg of the toxin per ml induced a nonspecific lysis of the target cells (data not shown). Moreover, depletion of $CD8^+$ T cells before incubation of the effector cells with the target cells and the CyaA224LCMV toxin totally abolished the killing, demonstrating that lysis by CTL of target cells incubated with CyaA224LCMV was mediated by $CD8^+$ T cells. These results therefore suggest that the recombinant ACT delivered the LCMV epitope into the MHC class I presentation pathway and thereby sensitized the target cells to killing by peptide-specific $CD8^+$ CTLs. It should be noted

that a significant lysis by CTLs was observed after incubation of the target cells with 10 μg or 1 μg of CyaA224LCMV per ml, which correspond, respectively, to 50 and 5 nM the LCMV peptide. A similar lysis of peptide-coated target cells by effector cells usually required 10 μg of free p118-126 peptide per ml, corresponding to 8.7 μM, a concentration more than 100-fold higher than that of CyaA224LCMV.

DISCUSSION

We have used one of the tagged toxins described here to test the hypothesis that peptides generated by proteolytic breakdown of the invasive AC can enter the MHC class I-restricted pathway of antigen presentation. It is well established that the uptake of soluble exogenous protein antigen by presenting cells does not allow its endogenous processing for presentation by MHC class I molecules (12). Entry into this pathway requires the presence of the peptides in the cytosolic compartment of the presenting cells, and in this respect, AC is a good candidate source of peptides by virtue of its cell-invasive capacity. The main finding reported here is that recombinant CyaA toxin bearing a $CD8^+$ T-cell LCMV epitope inserted into its catalytic domain sensitizes target cells for lysis by specific CTLs. This presentation of the LCMV epitope to MHC class I-restricted $CD8^+$ T cells is most likely a consequence of the proteolytic breakdown of the recombinant toxin in the cytosol of the target cells. Indeed, the wild-type toxin was previously shown to be rapidly proteolysed in human lymphocytes by an ATP-dependent mechanism (13).

The prerequisite for testing the recombinant CyaA toxins as a vehicle for delivering the foreign epitopes into antigen-presenting cells was that such recombinant toxins be similarly stable and functional as the wild-type protein. The results presented here show that full enzymatic, cytotoxic (invasive AC), and hemolytic activities were preserved in the epitope-tagged CyaA-LCMV proteins. Hence, the five sites previously identified in the AC domain of CyaA are also fully permissive for the insertion of heterologous peptides in the context of the entire toxin molecule. This observation enables the tagged toxins to be used as specific tools for the in vitro presentation of a foreign epitope to $CD8^+$ CTLs. The validity of the approach with one of the CyaA-LCMV proteins having been shown, it seems likely that recombinant CyaA molecules devoid of toxic AC activity are better candidates for delivery of foreign epitopes.

The CyaA-mediated delivery of the heterologous epitope of the LCMV virus into the MHC class I-restricted pathway raises the possibility that some peptides endogenous to AC may also enter this processing pathway. There is increasing evidence that not only the toxin but also the *Bordetella* cells themselves are capable of invading various eukaryotic cells (9). *B. pertussis* was found associated with alveolar macrophages of immunocompromised patients (6), and cell invasion and survival of the bacteria may be a mechanism for persistence of the pathogen in the host (6, 11, 31). It was reported that induction of cell-mediated immunity is required for effective immunization against *B. pertussis* (28), indicating the importance of cellular immune responses in the course of whooping cough pathogenesis. Indeed, induction of T-cell responses against pertussis toxin, and several other *B. pertussis* components, has been well documented in a mouse model and was also detected in an individual who had suffered from a pertussis infection (26). The role of AC in induction of cellular immunity against pertussis was, however, not studied. The results presented here indicate that induction of $CD8^+$ CTLs, specific for epitopes found in AC, may occur during pathogenesis by *Bordetella pertussis* and needs to be evaluated for its potential contribution to destruction of infected host cells and clearing of the pathogen.

In conclusion, we have generated a set of epitope-tagged ACTs that exhibit full cytotoxic activity. The use of such toxins, like those described in this study and those with ablated AC activity (cytotoxicity), would provide an attractive immunization strategy for delivery of foreign epitopes into antigen-presenting cells. Recently, the modified *Pseudomonas* exotoxin was shown to have the capacity to deliver MHC class I-restricted epitopes from influenza A virus to the MHC class I molecules and to sensitize target cells for lysis by peptide-specific CTLs (8). However, this toxin enters the cells by an endocytosis process involving a specific toxin receptor. In contrast, ACT does not require any specific receptor for entry and has the capacity to penetrate the plasma membrane of a wide variety of cell types directly.

The potential toxicity of the recombinant CyaAs could be a limitation to the in vivo use of this molecule. It must be emphasized, however, that upon repeated injections into rabbits and mice, no toxic effect of CyaA has been observed. In our experience, mice injected with 1.5 mg of wild-type or recombinant toxins per kg of body weight did not suffer of any adverse reaction. Thus, the capacity of recombinant CyaA molecules to elicit specific $CD8^+$ CTL responses in vivo can now be investigated in mice.

ACKNOWLEDGMENTS

We are grateful to Roy Gross for initial stimulating discussions.

This work was supported by grants from the Institut Pasteur, Centre National de la Recherche Scientifique (URA 1129), and the Human Frontier Science Program Organization. P.S. was supported by the Agence Nationale de Recherches sur le SIDA.

REFERENCES

1. **Aichele, P., H. Hengartner, R. M. Zinkernagel, and M. Schulz.** 1990. Antiviral cytotoxic T cell response induced by *in vivo* priming with a free synthetic peptide. J. Exp. Med. **171:**1815–1820.
2. **Barry, E. M., A. A. Weiss, I. E. Ehrmann, M. C. Gray, E. L. Hewlett, and M. S. Goodwin.** 1991. *Bordetella pertussis* adenylate cyclase toxin and hemolytic activities require a second gene, *cyaC*, for activation. J. Bacteriol. **173:** 720–726.
3. **Bellalou, J., H. Sakamoto, D. Ladant, C. Geoffroy, and A. Ullmann.** 1990. Deletions affecting hemolytic and toxin activities of *Bordetella pertussis* adenylate cyclase. Infect. Immun. **58:**3242–3247.
4. **Benz, R., E. Maier, D. Ladant, A. Ullmann, and P. Sebo.** 1994. Adenylate cyclase toxin of *Bordetella pertussis*: evidence for the formation of small ion-permeable channels and comparison with HlyA of *Escherichia coli*. J. Biol. Chem. **269:**27231–27239.
5. **Betsou, F., P. Sebo, and N. Guiso.** 1993. CyaC-mediated activation is important not only for toxic but also for protective activities of *Bordetella pertussis* adenylate cyclase-hemolysin. Infect. Immun. **61:**3583–3589.
6. **Bromberg, K., G. Tannis, and P. Steiner.** 1991. Detection of *Bordetella pertussis* associated with the alveolar macrophages of children with human immunodeficiency virus infection. Infect. Immun. **59:**4715–4719.
7. **Confer, D. L., and J. W. Eaton.** 1982. Phagocyte impotence caused by an invasive bacterial adenylate cyclase. Science **217:**948–950.
8. **Donelly, J. J., J. B. Ulmer, L. A. Hawe, A. Friedmann, X.-P. Shi, K. R. Leander, J. W. Shiwer, A. I. Oliff, D. Martinez, D. Montgomery, and M. A. Liu.** 1993. Targeted delivery of peptide epitopes to class I major histocompatibility molecules by a modified *Pseudomonas* exotoxin. Proc. Natl. Acad. Sci. USA **90:**3530–3534.
9. **Ewanowich, C. A., A. R. Melton, A. A. Weiss, R. K. Sherburne, and M. S. Peppler.** 1989. Invasion of HeLa 229 cells by virulent *Bordetella pertussis*. Infect. Immun. **57:**2698–2704.
10. **Fayolle, C., E. Deriaud, and C. Leclerc.** 1991. *In vivo* induction of cytotoxic T cell response by a free synthetic peptide requires $CD4^+$ T cell help. J. Immunol. **147:**4069–4073.
11. **Friedman, R. L., K. Nordensson, L. Wilson, E. T. Akporiaye, and D. E. Yocum.** 1992. Uptake and intracellular survival of *Bordetella pertussis* in human macrophages. Infect. Immun. **60:**4578–4585.
12. **Germain, R. N.** 1994. MHC-dependent antigen processing and peptide presentation: providing ligands for T lymphocyte activation. Cell **76:**287–299.

13. **Gilboa-Ron, A., A. Rogel, and E. Hanski.** 1989. *Bordetella pertussis* adenylate cyclase inactivation by the host cell. Biochem. J. **262:**25–31.
14. **Glaser, P., A. Elmaoglou-Lazaridou, E. Krin, D. Ladant, O. Bârzu, and A. Danchin.** 1989. Identification of residues essential for catalysis and binding of calmodulin in *Bordetella pertussis* adenylate cyclase by site-directed mutagenesis. EMBO J. **8:**967–972.
15. **Glaser, P., D. Ladant, O. Sezer, F. Pichot, A. Ullmann, and A. Danchin.** 1988. The calmodulin-sensitive adenylate cyclase of *Bordetella pertussis*: cloning and expression in *Escherichia coli*. Mol. Microbiol. **2:**19–30.
16. **Glaser, P., H. Sakamoto, J. Bellalou, A. Ullmann, and A. Danchin.** 1988. Secretion of cyclolysin, the calmodulin-sensitive adenylate cyclase-haemolysin bifunctional protein of *Bordetella pertussis*. EMBO J. **7:**3997–4004.
17. **Goodwin, M. S., and A. A. Weiss.** 1990. Adenylate cyclase toxin is critical for colonization and pertussis toxin is critical for lethal infection by *Bordetella pertussis* in infant mice. Infect. Immun. **58:**3445–3447.
18. **Gross, M. K., D. C. Au, A. L. Smith, and D. R. Storm.** 1992. Targeted mutations that ablate either the adenylate cyclase or hemolysin function of the bifunctional cyaA toxin of *Bordetella pertussis* abolish virulence. Proc. Natl. Acad. Sci. USA **89:**4898–4902.
19. **Hackett, M., L. Guo, J. Shabanowitz, D. F. Hunt, and E. L. Hewlett.** 1994. Internal lysine palmitoylation in adenylate cyclase toxin from *Bordetella pertussis*. Science **266:**433–435.
20. **Hedegaard, L., and A. Danchin.** 1985. The *cya* gene region of *Erwinia chrysanthemi* B374: organization and gene products. Mol. Gen. Genet. **201:** 38–42.
21. **Hewlett, E. L., M. C. Gray, I. E. Ehrmann, N. J. Maloney, A. S. Otero, L. Gray, M. Allietta, G. Szabo, A. A. Weiss, and E. M. Barry.** 1993. Characterization of adenylate cyclase toxin from a mutant of *Bordetella pertussis* defective in the activator gene, *cyaC*. J. Biol. Chem. **268:**7842–7848.
22. **Khelef, N., H. Sakamoto, and N. Guiso.** 1992. Both adenylate cyclase and hemolytic activities are required by *Bordetella pertussis* to initiate infection. Microb. Pathog. **12:**227–235.
23. **Ladant, D., P. Glaser, and A. Ullmann.** 1992. Insertional mutagenesis of *Bordetella pertussis* adenylate cyclase. J. Biol. Chem. **267:**2244–2250.
24. **Ladant, D., S. Michelson, R. S. Sarfati, A.-M. Gilles, R. Predeleanu, and O. Bârzu.** 1989. Characterization of the calmodulin-binding and of the catalytic domains of *Bordetella pertussis* adenylate cyclase. J. Biol. Chem. **264:**4015–4020.
25. **Mackman, N., J.-M. Nicaud, L. Gray, and I. B. Holland.** 1985. Genetical and functional organisation of the *Escherichia coli* haemolysin determinant 2001. Mol. Gen. Genet. **201:**282–288.
26. **Peppoloni, S., L. Nencioni, A. Di Tommaso, A. Tagliabue, S. Parronchi, S. Romagnani, R. Rappuoli, and M. T. De Magistris.** 1991. Lymphokine secretion nad cytotoxic activity of human $CD4^+$ T-cell clones against *Bordetella pertussis*. Infect. Immun. **59:**3768–3773.
27. **Plata, F., P. Langlade-Demoyen, T. Berbar, and P. Kourilsky.** 1987. Retrovirus antigens recognized by cytolytic T lymphocytes activate tumor rejection *in vivo*. Cell **48:**231–240.
28. **Redhead, K., J. Watkins, A. Barnard, and K. H. G. Mills.** 1993. Effective immunization against *Bordetella pertussis* respiratory infection in mice is dependent on induction of cell-mediated immunity. Infect. Immun. **61:**3190–3198.
29. **Sakamoto, H., J. Bellalou, P. Sebo, and D. Ladant.** 1992. *Bordetella pertussis* adenylate cyclase toxin: structural and functional independence of the catalytic and hemolytic activities. J. Biol. Chem. **267:**13598–13602.
30. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
31. **Saukkonen, K., C. Cabellos, M. Burroughs, S. Prasad, and E. Tuomanen.** 1991. Integrin-mediated localization of *Bordetella pertussis* within macrophages: role in pulmonary colonization. J. Exp. Med. **173:**1143–1149.
32. **Sebo, P., P. Glaser, H. Sakamoto, and A. Ullmann.** 1991. High-level synthesis of active adenylate cyclase toxin of *Bordetella pertussis* in a reconstructed *Escherichia coli* system. Gene **104:**19–24.
33. **Sebo, P., and D. Ladant.** 1993. Repeat sequences in the *Bordetella pertussis* adenylate cyclase toxin can be recognized as alternative carboxy-proximal secretion signals by the *Escherichia coli* α-haemolysin translocator. Mol. Microbiol. **9:**999–1009.
34. **Van Regenmortel, M. H. V., J. P. Briand, S. Muller, and S. Plaué.** 1988. Synthetic polypeptides as antigens, p. 95–127. Elsevier, Amsterdam.
35. **Weiss, A. A., E. L. Hewlett, G. A. Myers, and S. Falkow.** 1984. Pertussis toxin and extracytoplasmic adenylate cyclase as virulence factors of *Bordetella pertussis*. J. Infect. Dis. **150:**219–222.
36. **Zhou, X., U. M. Abdel Motal, L. Berg, and M. Jondal.** 1992. *In vivo* priming of cytotoxic T lymphocyte responses in relation to *in vitro* up-regulation of major histocompatibility complex class I molecules by short synthetic peptides. Eur. J. Immunol. **22:**3085–3090.