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Expression of a Mutant, Full-Length Form of Diphtheria Toxin in *Escherichia coli*

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A mutant, full-length form of diphtheria toxin was cloned into *Escherichia coli* K-12 and expressed under BL-1+EK-1 containment. A gene fragment encoding the catalytic domain of the toxin was subjected to oligonucleotide-directed mutagenesis to produce a three-base mutation of an active site residue; Glu-148 was thereby replaced by Ser. Ser-148 fragment A had less than 1% of the ADP-ribosyltransferase activity of wild-type fragment A. Next, the complementary portion of the toxin structural gene was spliced with the mutated DNA fragment downstream of codon 148 to produce a construct that encoded mutant whole toxin with Ser at position 148. The mutant toxin was indistinguishable from authentic diphtheria toxin by Western blot analysis, but was about 800-fold less cytotoxic than wild-type toxin for BS-C-1 cells. Evidence from subunit exchange experiments indicated that a substantial fraction of the mutant toxin contained a fully functional B moiety, capable of mediating the entry of wild-type fragment A into sensitive mammalian cells. This combination of approaches provides a means of applying recombinant DNA methods in *E. coli* to study structure-function relationships in whole diphtheria toxin.

Restrictions on the cloning of certain highly toxic proteins have retarded application of recombinant DNA methods to the study of these proteins. According to current National Institutes of Health guidelines, the intact structural gene for diphtheria toxin (DT) may be cloned only with special permission and under high-level containment (see Appendix F in reference 9). However, DNA fragments encoding certain nontoxic or hypotoxic fragments of the toxin have been cloned and expressed in *Escherichia coli* under BL-1+EK-1 conditions (6, 11). The use of cloned gene fragments has provided a satisfactory method to study catalytic function of the toxin, since this function resides in a proteolytic fragment (fragment A) that is stable and readily refolds into native form after denaturation. Receptor-binding and membrane-insertion functions have been more difficult to approach, however, because the region of the toxin containing these functions (fragment B) is unstable when separated from fragment A (4).

To circumvent problems in producing and purifying recombinant fragment B in *E. coli*, we sought a method to clone a mutant, hypotoxic form of whole DT in *E. coli*. Recent studies in this laboratory on the active site of the toxin provided a potential route to this end. Glutamic acid 148, which resides within fragment A, has been identified as an active-site residue by photolabeling with NAD (3), and the highly conservative Glu-to-Asp mutation (GAA→GAT) at position 148 has been shown to give a mutant fragment A with less than 1% of the ADP-ribosyltransferase activity of the wild type (10). We therefore reasoned that the three-base mutation, GAA→TCC, substituting Ser for Glu-148, would be essentially nonrevertible and should yield a form of fragment A with even lower ADP-ribosyltransferase activity than the Asp-148 form. One would then be able to splice a DNA fragment containing the Ser-148 mutation with one encoding the complementary portion of the toxin to yield a complete gene encoding a mutant form of intact toxin with very low toxic potential. We requested and received authorization to conduct these experiments from the Office of Recombinant DNA, National Institutes of Health, and from university and local regulatory units. The results are reported here.

MATERIALS AND METHODS

Materials. [*adenylate*-^{32}P]NAD was obtained from New England Nuclear Corp. The commercial sources for the enzymes used in this study and procedures used to purify DT and fragment A have been described (2, 10). BS-C-1 cells (African green monkey kidney cells; ATCC no. CCL 26) used in cytotoxicity experiments were maintained in minimum essential medium (Eagle) with nonessential amino acids, as outlined by the American Type Culture Collection.

Mutagenesis. Gene fragment F2 (Fig. 1) was subcloned from pRTF2B into the *Eco*RI-*Bam*HI site of M13mp19 (M13mp19::F2), and oligonucleotide-directed mutagenesis was performed as previously described (10). A 23-base synthetic oligonucleotide, 5′CTAGCGTTTCCTATATTAA TAAC3′, containing a three-base substitution (underlines), was used to change the GAA codon (glutamic acid) at amino acid position 148 to TCC (serine).

Construction of pBRDT-S148. An *Eco*RI-*Bam*HI fragment from M13mp19::F2 containing the oligonucleotide substitution changing Glu-148 to Ser was subcloned into the same restriction sites of pBR322, creating pRTF2B-S148. A *Cla*I-*Sal*I fragment from pUC8::DTB (pUC8::DTB is described below) was ligated into pRTF2B-S148 that had been digested with the same restriction enzymes. Transformants were digested with several combinations of restriction enzymes to identify those plasmids with the entire DT gene sequence. Two transformants were identified. This construction is illustrated in Fig. 2.

Preparation of cell extracts. Cells were prepared by incubating 1/100 dilutions of overnight cultures in L broth for 6 h at 37°C with shaking. Cells were pelleted and washed, and periplasmic extracts were prepared (10) and desalted over

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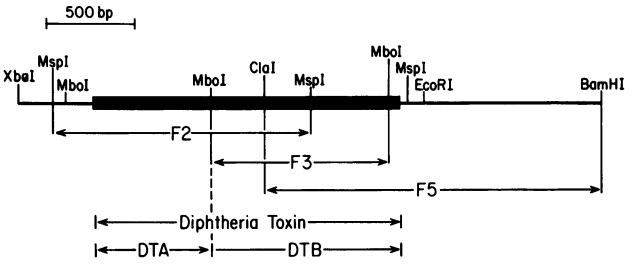

FIG. 1. Relationship of restriction fragments from corynephage β DNA to DT and the tryptic peptide fragments A and B. Shaded area, DT gene; bp, base pairs.

Sephadex G-50. The anti-DT immunoreactive protein in the desalted periplasmic extract was stable for several days at −20°C.

ADP-ribosyltransferase activity. Periplasmic extracts were digested with 3 μg of trypsin per ml for 30 min at 25°C in the presence of 200 μM NAD and were then chromatographed on Sephadex G-50 (bed volume > 10 times sample volume). Western blot analysis of the product showed that higher-molecular-weight toxin fragments had been completely digested to DTA. ADP-ribosyltransferase activity of the trypsin-treated, desalted extract was assayed as follows. The reaction mixture contained 50 mM Tris hydrochloride (pH 8.2), 1 mM EDTA, 40 mM dithiothreitol, 14 nM ^{32}P-NAD (specific activity, 787 Ci/mmol), wheat germ elongation factor 2, and periplasmic fraction in a final volume of 100 μl. After 30 min at 37°C, incorporation of label into trichloroacetic acid (TCA)-precipitable material was determined as previously described (10).

Cytotoxicity assay. Confluent lawns of BS-C-1 cells in 24-well plastic culture dishes were incubated in 0.5 ml of fresh culture medium containing the indicated concentration of authentic DT or DT–Ser-148. After 5 h, the medium was removed and cells were incubated in 0.5 ml of assay medium containing leucine, at 1/20 the concentration present in the original medium, and 1 μCi of [^{3}H]leucine per ml. After 1 h, cells were washed twice with cold 10% TCA and once with phosphate-buffered saline and dissolved in 0.4 ml of 0.1 N NaOH. Incorporation of leucine into TCA-precipitable material was determined by liquid scintillation.

Exchange of wild-type fragment A for mutant A in DT–Ser-148. The method used to exchange wild-type for mutant fragment A in DT–Ser-148 was similar to that of Uchida et al. (13). A 100-μl sample of trypsin-treated pBRDT-S148 periplasm was reduced with 20 mM dithiothreitol and incubated at 37°C for 10 min. (Figure 5, lane C, shows a Western blot of the trypsin-treated periplasm of pBRDT-S148 used in this experiment.) Concurrently a 7.5-fold molar excess of authentic fragment A was reduced under the same conditions, and the two preparations were mixed and dialyzed overnight at 5°C in 50 mM Tris hydrochloride (pH 7.6). The dialysates were assayed for cytotoxic activity and for immunoreactive material by densitometry of Western blots probed with ^{125}I-anti-DT.

Construction of pUC8::DTB. The complete B fragment of DT was cloned into pUC8 as a fusion protein. The F3 gene fragment of DT (Fig. 1) was isolated from a *Sau* 3A digest of pRTF3 and cloned into the *Bam*HI site of pUC8. One transformant (pUC8::F3) that possessed the desired *Sau*3A insert orientated in frame, with respect to the β-galactosidase gene, was identified. The F5 DNA fragment of the DT gene (Fig. 1) was cloned into the *Cla*I-*Bam*HI sites of pBR322, yielding pRTF5 (R. Tweten and R. J. Collier, unpublished results). A *Cla*I/*Sal*I fragment from pRTF5 (1), which encoded DNA for the carboxyl-terminal region of DT, was ligated into pUC8::F3 which had been digested with the same enzymes. One transformant (pUC8::DTB-long) was isolated which had a restriction digest profile that indicated the presence of the complete gene for fragment B. Corynebacterial DNA outside the DT structural gene was removed by subcloning an *Eco*RI restriction fragment containing information for the entire B moiety of DT from pUC8::DTB-long into the *Eco*RI site of pUC8, yielding pUC8::DTB (Fig. 2).

RESULTS

The F2 gene fragment, which encodes the toxin signal peptide, all of the fragment A moiety, and 189 residues of the B moiety of DT (5), was subcloned into M13mp19 and subjected to directed mutagenesis with the synthetic oligonucleotide 5′ CTAGCGTTTCCTATATTAATAAC 3′. A three-base substitution (GAA→TCC; underlined) was thereby made in the codon of Glu-148 to change this residue to serine. Mutants were identified by colony blot hybridization (7), with the mutagenic oligonucleotide which had been

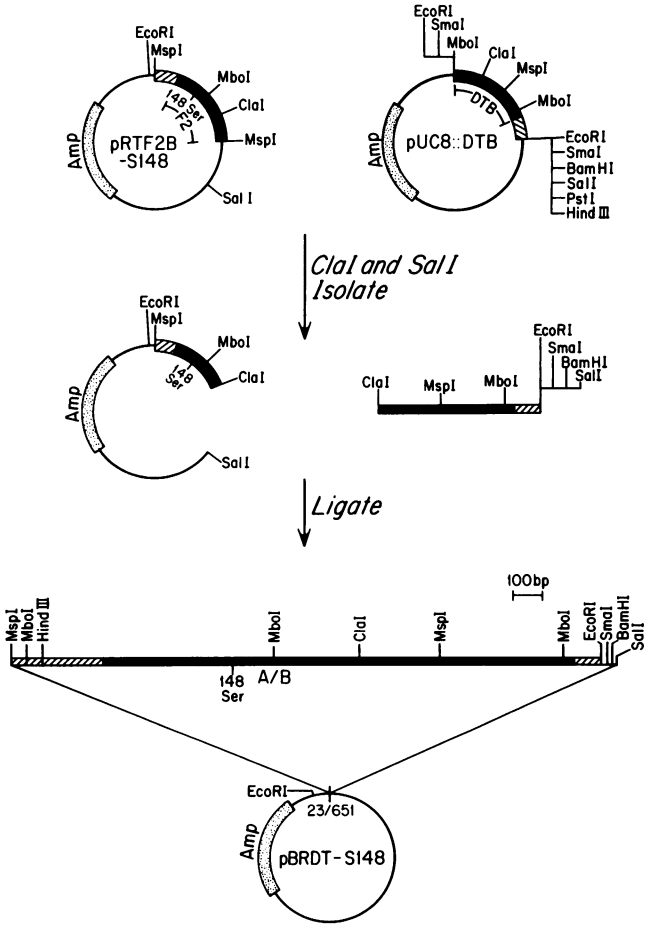

FIG. 2. Protocol for construction of pBRDT-S148. Shaded area, DT structural gene; hatched area, flanking regions of corynephage β DNA. Bases 23 through 623 of pBR322 were deleted in the course of inserting the DT gene. Plasmids are not drawn to scale, except the DT gene of pBRDT-S148 (bottom), where bar is equivalent to 100 base pairs.

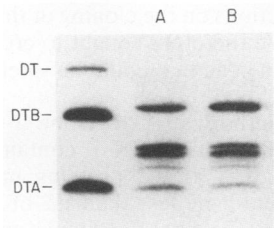

FIG. 3. Western blot analysis of periplasmic extracts from cells containing pRTF2B (A) or pRTF2B-S148 (B). Periplasmic extracts were electrophoresed in 12.5% polyacrylamide gels in the presence of 1.0% sodium dodecyl sulfate and 2.5% β-mercaptoethanol. Immunoreactive peptides were detected by Western blot analysis with ^{125}I-anti-DT (10). The largest immunoreactive peptide in lanes A and B represents the product of toxin gene fragment F2 (M_r, 43,000); the lower-molecular-weight immunoreactive peptides presumably represent degradation products. DT (M_r, 58,342), fragment B (DTB; M_r, 37,198), and fragment A (DTA; M_r, 21,164) were used as molecular weight markers.

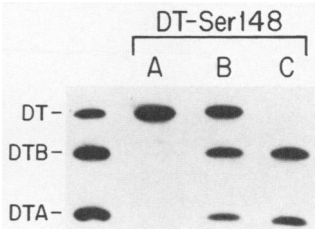

FIG. 5. Western blot analysis of periplasmic extracts from cells containing pBRDT-S148. Periplasmic extract was incubated alone (A), or with 1.0 μg (B) or 3.0 μg (C) of trypsin per ml, for 30 min at 25°C in the presence of 200 μM NAD. Digestion was stopped by adding soybean trypsin inhibitor (50 μg/ml, final concentration), and the samples were electrophoresed on 12.5% polyacrylamide gels in the presence of 1.0% sodium dodecyl sulfate and 2.5% β-mercaptoethanol. Immunoreactive peptides were detected by Western blot analysis with ^{125}I-anti-DT (10). DTB, Fragment B; DTA, fragment A.

end labeled with ^{32}P, and the three-base substitution was confirmed by dideoxy sequencing (8). The F2 gene fragment containing the serine mutation was religated into pBR322, creating pRTF2B-S148.

Periplasmic extracts of *E. coli* JM103 containing pRTF2B and pRTF2B-S148 gave identical patterns of immunoreactive peptides as detected by Western blot analysis with labeled anti-DT (Fig. 3). Such extracts were subjected to mild digestion with trypsin, under conditions that converted all higher-molecular-weight toxin peptides to the corresponding wild-type or Ser-148 forms of fragment A, and were then assayed for ADP-ribosyltransferase activity. The activity of pRTF2B-S148 mutant extract, either untreated or trypsin treated, was below the limit of detection of our system (±0.3% of the wild-type activity; Fig. 4) and therefore well below 1% of wild-type activity. The trypsin-treated periplasmic extract from the pRTF2B-S148 strain did not contain inhibitors of ADP-ribosyltransferase activity, as shown by assaying mixtures of the two extracts (data not shown).

A *Cla*I-*Sal*I restriction fragment encoding the complementary, carboxyl-terminal region of DT was isolated from pUC8::DTB and ligated into pRTF2B-S148 which had been digested with the same enzymes. Before ligation, pRTF2B-S148 was treated with calf intestinal alkaline phosphatase to prevent fragment religation (7). The ligation mixture was transformed into *E. coli* JM103. Two identical transformants were isolated which possessed the entire, mutant toxin gene (DT–Ser-148) as determined by restriction site analysis, and one was chosen for further study.

Periplasmic extracts of *E. coli* JM103 containing DT–Ser-148 showed a single immunoreactive peptide with the same molecular weight as authentic DT on Western blots probed with anti-DT (Fig. 5). Neither fragment A nor fragment B was detected on Western blots of sodium dodecyl sulfate-polyacrylamide gels electrophoresed in the presence of beta-mercaptoethanol, indicating that the trypsin-sensitive region of the mutant toxin was "unnicked." Like intact DT, the unnicked immunoreactive protein in DT–Ser-148 periplasmic extracts could be cleaved with trypsin to two immunoreactive peptides having molecular weights identical to those of fragment A and fragment B (Fig. 5).

Less than 5% of the total DT–Ser-148 immunoreactive protein produced in *E. coli* JM103 was located in the culture medium, indicating that this strain did not excrete the DT–Ser-148 protein (data not shown). *E. coli* does not

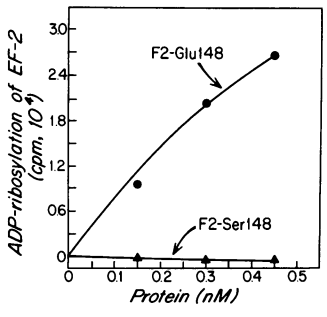

FIG. 4. Titration of wild-type and mutant peptides in ADP-ribosyltransferase assay. Periplasmic extracts from cells containing pRTF2B (●) or pRTF2B-S148 (▲) were assayed for ADP-ribosyltransferase activity after mild trypsin digestion, as described in the text. The background incorporation was 4,290 cpm, which was subtracted from experimental values. Enzyme concentration was estimated by radioimmunoassay. The standard deviation of the measurements of activity of pRTF2B-S148 extracts was ±0.3% the activity of pRT-F2B extracts.

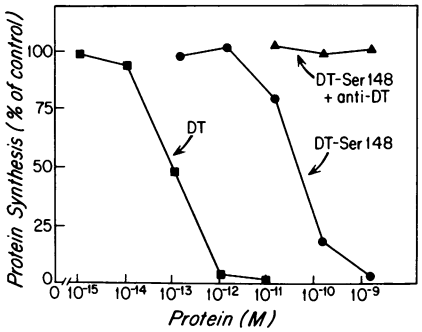

FIG. 6. Inhibition of protein synthesis in BS-C-1 cells by DT–Ser-148. BS-C-1 cells were incubated with 0.5 ml of fresh medium containing the indicated concentration of DT (■), DT–Ser-148 (●), or DT–Ser-148 preincubated with 5.0 μg of anti-DT antibody (▲). After 5 h, medium was removed, and cells were assayed for the ability to incorporate leucine into TCA-precipitable material as described in the text. Control cells incorporated 10,600 cpm. Toxin protein concentration was estimated by radioimmunoassay.

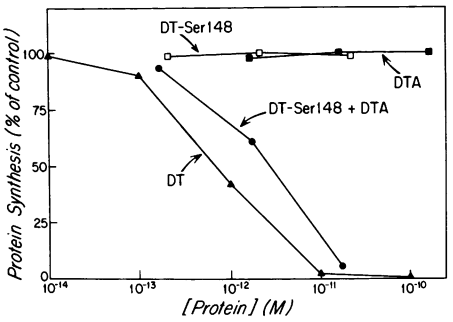

FIG. 7. Inhibition of protein synthesis in BS-C-1 cells by DT–Ser-148 subjected to subunit exchange with wild-type fragment A. BS-C-1 cells were incubated with 0.5 ml of fresh medium containing the indicated concentration of DT (▲), DT–Ser-148 + fragment A exchange mixture (●), DT–Ser-148 (□), or fragment A (■). After 5 h, medium was removed, and cells were tested for the ability to incorporate leucine into TCA-precipitable material, as described in the text. Control cells incorporated 15,200 cpm into TCA-precipitable material. Protein concentration was estimated by radioimmunoassay.

excrete either the F1 or F2 gene product into the culture medium (6, 11).

Ser-148 toxin in periplasmic extracts was about 800-fold less toxic than native DT for BS-C-1 cells (Fig. 6). Preincubation of the periplasmic extract with anti-DT eliminated cytotoxicity for BS-C-1 cells. The same level of activity was detected in preparations from two independent transformants, and it is therefore unlikely that the toxicity observed was due to contaminating native toxin. Periplasmic extracts from *E. coli* JM103 carrying pBR322 without the DT–Ser-148 insert did not inhibit protein synthesis in BS-C-1 cells and did not reduce the cytotoxic potential of authentic DT (data not shown).

The simplest interpretation of our results was that the diminution of cytotoxicity of DT–Ser-148 relative to that of authentic toxin was solely attributable to the reduction in ADP-ribosyltransferase activity. To determine whether the fragment B portion of DT–Ser-148 was functional, we performed an experiment to measure the toxicity of a hybrid protein formed by exchanging wild-type fragment A for the mutant fragment A of Ser-148 toxin in periplasmic extracts. The periplasmic extract was digested with trypsin to nick the Ser-148 toxin (Fig. 5, lane C). The extract was reduced with dithiothreitol and mixed with a 7.5-fold molar excess of native fragment A that had been concurrently reduced with the same reagent. The protein mixture was then dialyzed at 5°C overnight. Cytotoxic activity of the dialysate was found to have increased about 250-fold, to a level one-third that of pure, nicked DT (Fig. 7). Neither native fragment A nor nicked Ser-148 toxin alone, at the concentrations present in the subunit exchange mixture, was cytotoxic for BS-C-1 cells.

DISCUSSION

Although several important mutants in the DT structural gene have been isolated by random mutagenesis of corynephage β (12, 14), the lack of a convenient genetic transfer system in *Corynebacterium diphtheriae* and the lack of easily selectable phenotypic markers for mutations that affect the cytotoxicity of DT have hampered the genetic analysis of the toxin's structure-function relationships. Also, the whole toxin was not been cloned to date in *E. coli* because of restrictions on the cloning of this and other highly toxic proteins. We therefore sought to create a mutant form of the whole toxin gene that could be safely constructed and manipulated in *E. coli*.

We received authorization to clone a mutant full-length form of DT under BL-1+EK-1 containment, under the following conditions: (i) the proteins produced from the altered gene should have less than 1% of the toxicity of the wild-type toxin; (ii) the cloned sequence encoding the altered fragment A should differ from wild-type A by a deletion or change of 3 base pairs in one codon; and (iii) the bacterial host should lack and be monitored for suppressors which might result in expression of functional toxin.

Serine was chosen as a replacement for Glu-148 for two major reasons. First, a codon differing in all three bases could be used to effect the mutation. Second, the serine replacement represented a greater alteration of side-chain structure than the aspartic acid substitution, which was already known to reduce catalytic activity by a factor of at least 100. Also, since the serine side chain is not bulky and retains slight polar character, it was unlikely to cause major perturbations in folding.

The ADP-ribosyltransferase activity of the Ser-148 form of fragment A was undetectable by our standard assay, which placed the level well below 1% of wild-type A. With a more sensitive assay developed recently in our laboratory (K. Reich and R. J. Collier, unpublished data), we have found that Ser-148 fragment A does have finite enzymic activity, estimated at 3 to 4 orders of magnitude below that of wild type. More precise measurements, as well as determination of changes in specific kinetic parameters, will be possible when the protein is obtained in pure form and in larger amounts.

The Ser-148 mutant of whole toxin was about 800-fold less cytotoxic than native DT. The residual toxicity of the mutant toxin, which is neutralizable by anti-DT antibody, apparently reflects the finite ADP-ribosyltransferase activity of the Ser-148 form of fragment A. To obtain an indication of whether the B domain of the mutant toxin was properly folded in *E. coli*, we conducted an experiment in which nicked, mutant toxin was incubated with an excess of wild-type fragment A under conditions known to facilitate the exchange of A. The fact that toxicity increased to a level approximately one-third that of pure, authentic toxin suggests that at least this substantial fraction of the mutant toxin contains functional B. These results must be viewed in light of the facts that the exchange may have been incomplete and that residual Ser-148 toxin may have competed for receptor binding in the toxicity assay. Thus it may well be that most or all of the Ser-148 toxin produced in *E. coli* folded correctly and contained fully functional B. Regardless, the evidence supports the notion that the diminution in toxicity of Ser-148 toxin, relative to the wild type, resulted largely or solely from reduction in ADP-ribosyltransferase activity. More accurate determinations will be possible once we have isolated the Ser-148 toxin in larger quantity and in pure form.

The studies reported here provide a new means of genetically manipulating and analyzing a mutant, full-length DT gene in *E. coli*. This should facilitate more extensive analysis of structure-function relationships in the toxin and possibly also development of alternative vaccines.

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