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Production of a Complete Binary Toxin (Actin-Specific ADP-Ribosyltransferase) by *Clostridium difficile* CD196

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A *Clostridium difficile* isolate was found to produce an actin-specific ADP-ribosyltransferase (CDT) homologous to the enzymatic components of *Clostridium perfringens* iota toxin and *Clostridium spiroforme* toxin (M. R. Popoff, E. J. Rubin, D. M. Gill, and P. Boquet, Infect. Immun. 56:2299–2306, 1988). The CDT locus from *C. difficile* CD196 was cloned and sequenced. It contained two genes (*cdtA* and *cdtB*) which display organizations and sequences similar to those of the iota toxin gene. The deduced enzymatic (CDTa) and binding (CDTb) components have 81 and 84% identity, respectively, with the corresponding components of iota toxin. CDTa and CDTb induced actin cytoskeleton alterations similar to those caused by other clostridial binary toxins. The lower level of production of binary toxin by CD196 than of iota toxin by *C. perfringens* was related to a lower transcript level, possibly due to a promoter region different from that of iota toxin genes. The *cdtA* and *cdtB* genes have been detected in 3 of 24 clinical isolates examined, and *cdtB* alone was found in 2 additional strains. One strain (in addition to CD196) was shown by Western blotting to produce CDTa and CDTb. These results indicate that some *C. difficile* strains synthesize a binary toxin that could be an additional virulence factor.

Clostridium difficile is the causative agent of pseudomembranous colitis and is also implicated in about 20% of all cases of antibiotic-associated diarrhea and colitis. The main *C. difficile* virulence factors are toxins A and B (ToxA and ToxB), which are large, single-chain protein toxins (308 and 270 kDa, respectively) (2, 19). ToxA and ToxB are both cytotoxic for cultured cells, but ToxB is much more potent than ToxA. Both toxins disrupt the actin cytoskeleton by monoglycosylation of regulatory proteins belonging to the Rho family, which are involved in the control of the actin polymerization (9, 10).

It has been shown that, in addition to ToxA and ToxB, 1 *C. difficile* strain (CD196), of 15 strains tested, produces an actin-specific ADP-ribosyltransferase called CDT (17). CDT is related to the clostridial binary toxins, which include iota toxin from *Clostridium perfringens* type E, *Clostridium spiroforme* toxin, and C2 toxin from *Clostridium botulinum* C and D (15, 17). These toxins consist of two independent protein chains not linked by either covalent or noncovalent bonds (6). The binding component (about 100 kDa) recognizes a cell surface receptor and allows the internalization of the enzymatic component into the cytosol (20). The enzymatic component (about 45 kDa) catalyzes the ADP-ribosylation reaction of monomeric actin and induces a disorganization of the cytoskeleton (1). Interestingly, CD196 was found by biological activity screening (17) to produce only the enzymatic component (CDTa, previously called CDT) and no binding component (CDTb). CDTa was also shown (17) to be immunologically related to the enzymatic components of iota toxin (Ia) and *C. spiroforme* toxin (Sa) but not to that of C2 toxin. Moreover, these previous studies have shown that CDTa can associate with the binding component of iota toxin (Ib) or *C. spiroforme* toxin (Sb) to form fully active toxins, while the C2 toxin binding component is inefficient for the internalization of CDTa into the cells (15, 16).

In this study, we report that *C. difficile* CD196 contains both the enzymatic- and the binding-component genes (*cdtA* and *cdtB*, respectively), that both components of this toxin are produced, and that this binary toxin is cytotoxic for cells. In addition, we investigated the distribution of the *cdtA* and *cdtB* genes in clinical isolates by DNA-DNA hybridization.

MATERIALS AND METHODS

Bacterial strains and plasmids. *C. difficile* CD196 (17) and *C. perfringens* NCIB10748 (13) were grown in broth containing Trypticase (30 g/liter), yeast extract (20 g/liter), glucose (5 g/liter), and cysteine-HCl (0.5 g/liter) (pH 7.2) under anaerobic conditions. *Clostridium* DNA was extracted and purified as previously described (13).

Plasmid pUC19 (Appligene, Strasbourg, France) was used for cloning in *Escherichia coli* TG1.

DNA techniques. Ligation, transformation, and preparation of plasmid DNA from *E. coli* were conducted as described previously (18). DNA was sequenced by the dideoxy chain terminator procedure with a Sequenase kit (United States Biochemical Corp., Cleveland, Ohio).

RNA isolation and RNA dot blots. Total RNA was extracted from *C. difficile* and *C. perfringens* mid-exponential-phase cultures by using Trizol (Gibco BRL, Cergy Pontoise, France). The bacterial pellet from 10 ml of culture (optical density, about 1) was washed twice in distilled water and suspended in 200 μl of 10 mM Tris-HCl (pH 7)–10 mM EDTA–20% sucrose containing 1 mg of lysozyme. The mixture was incubated for 30 min at 37°C and centrifuged. The pellet was suspended in 1 ml of Trizol and incubated for 5 min at room temperature. The following steps of RNA purification were done according to the manufacturer's recommendations.

Serial dilutions of total RNA in 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) were transferred onto Hybond N+ membranes (Amersham, Paris, France) and hybridized at 60°C for 2 h by using rapid hybridization buffer (Amersham) and PCR-amplified fragments (positions 42 to 1969 for the Ia probe and 3457 to 3865 for the Ib probe) (13) ^{32}P labeled with the Megaprime kit (Amersham). The membranes were washed in 0.1× SSC–0.1% sodium dodecyl sulfate (SDS) at 60°C and exposed to X-ray films. For Northern blot analysis, total RNA was separated by agarose-formaldehyde gel electrophoresis and then was transferred onto a membrane.

Polyacrylamide gel electrophoresis (PAGE) and immunoblotting procedure. Proteins were precipitated from the supernatant (100 ml) of an overnight culture of CD196 by 70% ammonium sulfate saturation. The precipitate was collected by centrifugation, dissolved in 2 ml of distilled water, and dialyzed against 10 mM Tris-HCl, pH 7. The corresponding bacterial pellet was washed twice in distilled water, suspended in 2 ml of 10 mM Tris-HCl, pH 7, containing 10 mM benza-

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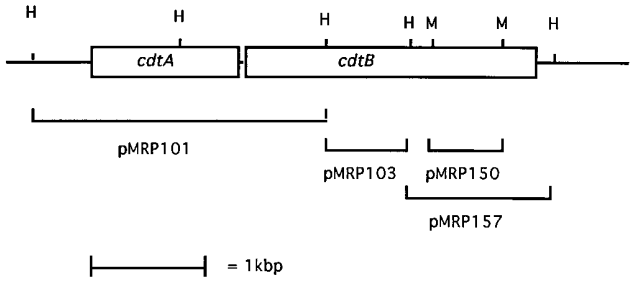

FIG. 1. Strategy for cloning of the *cdtA* and *cdtB* genes, encoding the enzymatic component (CDTa) and the binding component (CDTb), respectively. H, *Hin*dIII; M, *Mbo*I.

midine, and sonicated for 5 min. The mixture was centrifuged (15,000 × *g*), and then the supernatant was collected and designated the cellular extract.

The binding components of iota toxin (Ib) and *C. spiroforme* toxin (Sb) were purified as previously described (13, 14).

The immunoblotting procedure of Burnette (5) was used. Proteins separated by 0.1% SDS–10% PAGE were transferred electrophoretically to nitrocellulose sheets (Hybond C; Amersham). The nitrocellulose sheets were incubated for 1 h in phosphate-buffered saline containing 5% dried milk and then incubated overnight at room temperature with a 1:400 dilution of immunopurified Ia or Ib rabbit antibodies (13). Bound antibodies were detected with peroxidase-labeled protein A and a chemiluminescence kit provided by Amersham.

Cytotoxicity assay. Cells were cultivated in Dulbecco modified Eagle medium supplemented with 5% fetal bovine serum. Vero (African green monkey kidney) cells were transferred into the wells of a 96-well Falcon tissue culture plate (Becton Dickinson Labware, Oxnard, Calif.) and grown for 24 h to form monolayers. Serial twofold dilutions of samples in a 100-μl final volume were added to the monolayers. The cells were inspected 24 h after incubation for morphological alteration. The actin cytoskeleton was visualized by immunofluorescence with fluorescein isothiocyanate-phalloidin (1 μg/ml; Sigma Chemical Co., L'Isle d'Abeau, France) as previously described (8).

Nucleotide sequence accession number. The nucleotide sequence reported in this paper has been submitted to the GenBank Data Library with accession number L76081.

RESULTS

Cloning of the *cdtA* and *cdtB* genes. The iota toxin genes (*iap* and *ibp*) encoding the Ia and Ib components from *C. perfringens* E have been characterized previously (13). The recombinant plasmid pMRP105 containing most of the *iap* and *ibp* genes (13) was found to hybridize with total DNA extracted from *C. difficile* CD196 (data not shown). Therefore, this plasmid was used as a probe to clone two DNA fragments (pMRP101 and pMRP103) from CD196 (Fig. 1). The recombinant plasmids pMRP150 and pMRP157 were identified by screening a library of *C. difficile* CD196 DNA by hybridization with a probe consisting of an amplified fragment of the *ibp* gene (positions 4382 to 5502) (13). The sequenced region of 4,277 bp contained two open reading frames, named *cdtA* and *cdtB* (Fig. 1).

***cdtA* and deduced amino sequence.** The *cdtA* gene extends from positions 105 to 1487 and is preceded by a consensus ribosome binding site (GGGAGGG) seven nucleotides upstream from the initiation codon (Fig. 2). The DNA coding region of *cdtA* has 84.6% identity with the *iap* gene. However, the 104 nucleotides upstream from the *cdtA* gene, which probably contain the promoter region, are less closely related (60% identity) to the corresponding upstream region preceding the *iap* gene. The inverted repeat starting at the transcriptional start site, as well as the three consensus binding sequences for an Hpr-like regulatory protein that are characteristic of the *iap* promoter (14), was not found in the region upstream from *cdtA*. However, an inverted repeat able to form a stem-loop structure ($\Delta G = -8.70$ kcal/mol) was identified as a sequence 5′ to the *cdtA* gene (Fig. 2).

The deduced protein (CDTa) is predicted to be 460 amino acids long, which corresponds to the length of the previously described CDT (17). The program described by Klein et al. (11) predicts that the 43 N-terminal amino acids of CDTa will form a transmembrane segment, and this region probably constitutes a signal peptide. By analogy with Ia, the proteolytic cleavage site for this signal peptide is probably Lys-42–Val-43. This assumption predicts that the mature CDTa protein should be 418 amino acids long and have a molecular weight of 47,986, which is in reasonable agreement with the estimated size of the purified native protein (43 kDa) (17). The amino acid sequence of mature CDTa has 84.3% identity with that of Ia as well as a similar molecular weight (47,588 for mature Ia) but has a substantially different pI (9.34 for CDTa versus 5.44 for Ia). The residues forming the active site of Ia, which apparently consists of a β-strand and an α-helix flanked by three amino acids (Arg-295, Glu-378, and Glu-380) that are essential for the catalytic activity (12, 21), are conserved in CDTa (stretch from Phe-336 to Ile-354 and residues Arg-296, Glu-379, and Glu-381) (Fig. 2).

***cdtB* and deduced amino acid sequence.** The open reading frame found downstream of the *cdtA* gene is similar to the *ibp* gene (84.1% identity) and therefore was named the *cdtB* gene. A consensus ribosome binding site (GGAGG) is localized 7 bp upstream of the initial start codon. The intergenic region (52 bp) found between *cdtA* and *cdtB* and the 106 bp downstream of *cdtB* are weakly similar (61.5 and 40.8% identity, respectively) to the corresponding regions flanking the *ibp* gene. A Rho-independent terminator sequence ($\Delta G = -16.1$ kcal/mol) was identified downstream from the *cdtB* gene, whereas no potential stem-loop structure was evident in the 3′ part of the *ibp* gene.

The 42 N-terminal residues encoded by the *cdtB* open reading frame are predicted to form a transmembrane segment and display the feature of a signal peptide (13). A common cleavage site (Lys-Glu) is found between the putative signal peptide and the precursor form of CDTb and Ib. The deduced CDTb component consists of 876 amino acids (predicted molecular mass, 98,895 Da), with 81.2% identity to the precursor Ib protein. The activated Ib form is released from the Ib precursor by proteolysis between Ala-212 and Ala-213 (13). This cleavage site is not conserved in CDTb, but another putative proteolytic site (Lys-209–Leu-210) is found in the corresponding position (Fig. 2). CDTb and Ib display a conserved ATP/GTP binding site (GXXXXGK[T/S]) and a conserved hydrophobic sequence (Leu-292 to Ser-309 in Ib and Leu-293 to Ser-310 in CDTb) predicted to form a transmembrane segment (11, 13) which could be involved in the translocation of the toxin across the cell membrane.

***cdtA* and *cdtB* expression in *C. difficile* CD196.** The surprising feature emerging from the genetic analysis of CD196 is the presence of a complete gene for a binding component homologous to Ib, although a previous study could not demonstrate a functional binding component in crude culture supernatant of this strain (17). To detect possible production of this putative binding component, immunoblotting experiments were performed with CD196 culture supernatant and cell extracts (Fig. 3). CDTa and CDTb were detected by immunoblotting of CD196 culture supernatant concentrated by ammonium sulfate precipitation (about 40-fold) but not in the bacterial extracts. Thus, both components are produced and secreted, but at low levels (less than 1% of the entire amount of secreted proteins as assayed by immunoblotting) (Fig. 3).

The immunoreaction of CDTb with anti-Ib antibodies which also recognize Sb confirms the close relation of CDTb to Ib and Sb, as previously reported for CDTa, Ia, and Sa (15, 17).

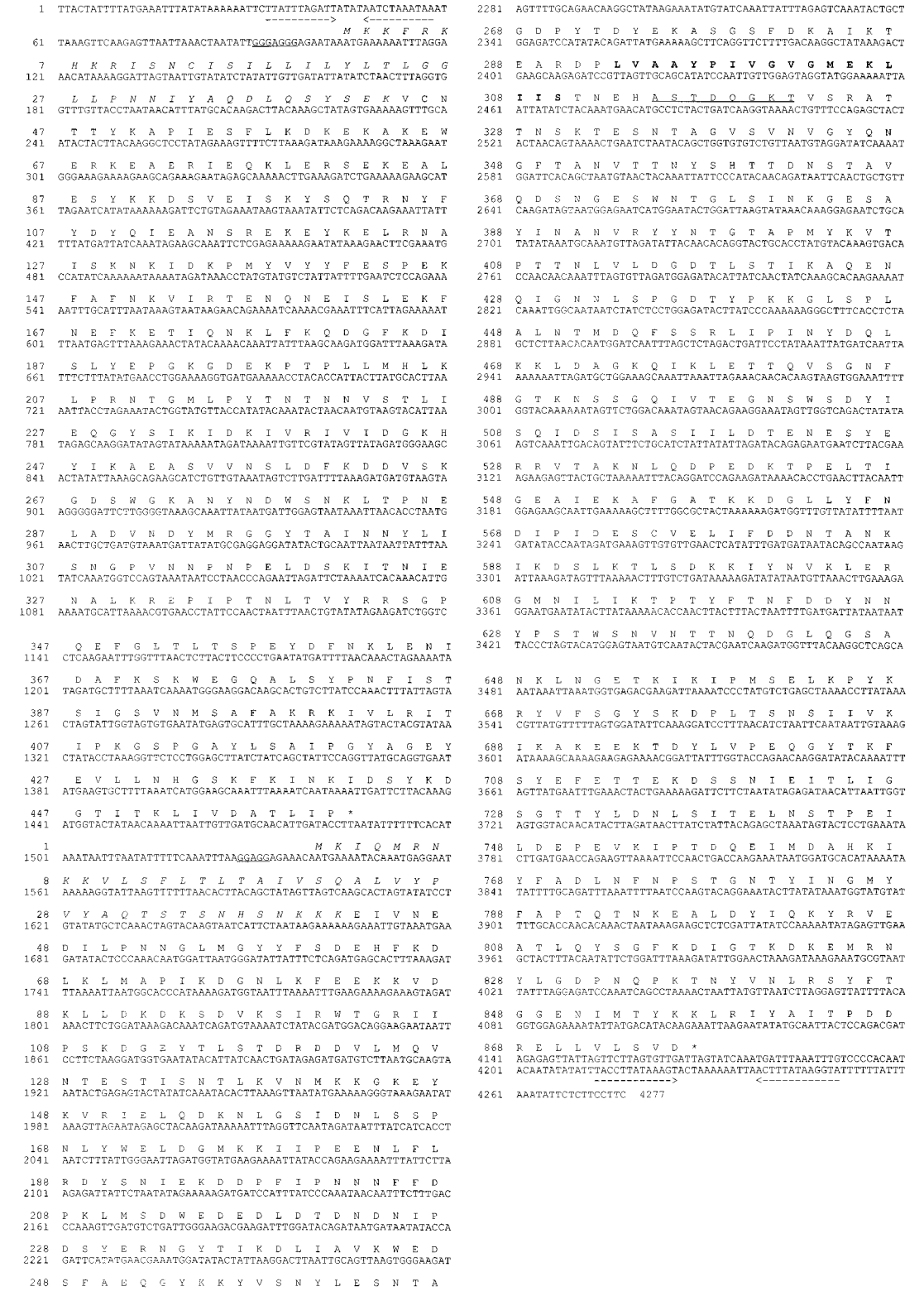

FIG. 2. Nucleotide sequence and deduced amino acid translation of the *cdtA* and *cdtB* genes. The putative ribosome binding sites are underlined. The predicted signal peptides are italicized. Stop codons are indicated by asterisks, and inverted repeats are indicated by dashed arrows beneath the sequences. The predicted CDTb transmembrane segment is in boldface, and the putative ATP/GTP binding motif is underlined.

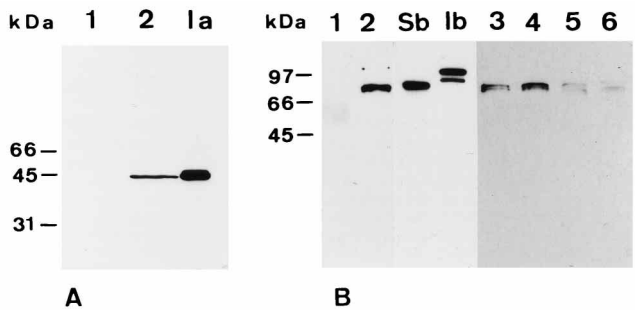

FIG. 3. Production of CDTa and CDTb by *C. difficile* CD196 shown by Western blotting. Samples of cell extract (50 μg of total protein [lane 1]), concentrated culture supernatant (50 μg of total protein [lane 2]), purified Ia (130 ng [lane Ia]), Sb (200 ng [lane Sb]), Ib (70 ng [lane Ib]) (the upper band corresponds to the precursor, and the lower band corresponds to the mature form), and culture supernatant (50 μg of total protein) treated with trypsin at concentrations of 60 ng/ml (lane 3), 300 ng/ml (lane 4), 3 μg/ml (lane 5), and 30 μg/ml (lane 6) for 20 min at room temperature were separated by 0.1% SDS–10% PAGE, transferred to a nitrocellulose membrane, and probed with anti-Ia antibodies (A) and anti-Ib antibodies (B).

The size of CDTb (75 kDa) estimated by SDS-PAGE (Fig. 3) is identical to that of mature Sb (75 kDa) and slightly smaller than that of mature Ib (84 kDa) (Fig. 3). This is in agreement with the predicted Lys-209–Leu-210 proteolytic cleavage site, which would give a protein with a deduced molecular mass of 74,707 Da.

CDTa and CDTb activity was tested in Vero cells by trypsinizing concentrated culture supernatant to proteolyze ToxA and ToxB and to activate CDTb. The smallest concentration of total proteins required to induce a cytotoxic effect which can be neutralized by anti-Ib antibodies was 20 μg/ml, in contrast to 0.5 μg/ml for *C. perfringens* NCIB10748 (Fig. 4). Thus, CDTa and CDTb production is apparently about 40-fold less than that of iota toxin in *C. perfringens* E. The Vero cell morphological alterations caused by CDTa and CDTb included rounding and depolymerization of actin filaments as visualized with fluorescein isothiocyanate-phalloidin (Fig. 4) and were similar to those previously observed with iota toxin and *C. spiroforme* toxin (15, 16).

To determine whether the CDTb component produced by CD196 is the active form, a concentrated culture supernatant sample was stored at −20°C for 4 days and then thawed; some aliquots were trypsinized and tested for cytotoxicity. Freezing at −20°C usually inactivates ToxA and ToxB (unpublished data). Trypsinized culture supernatant was cytotoxic (Fig. 4), whereas the untrypsinized samples were not, indicating that secreted CDTb required trypsinization for activity. SDS-PAGE and immunoblotting of trypsinized culture supernatant showed a processing of CDTb into a slightly more mobile band (about 73 kDa) (Fig. 3). The presence of CDTb in CD196 culture supernatant probably results from a proteolytic cleavage of the presumed precursor, with complete activation seeming to require an additional cleavage by trypsin which removes a small peptide.

To determine if the apparent low level of production of the CDTa and CDTb components is due to a low level of transcription in CD196, RNA dot blot experiments were performed (Fig. 5). The amounts of *cdtA* and *cdtB* gene transcripts from CD196 were significantly lower than those of the *iap* and *ibp* genes from *C. perfringens* NCIB10748. This suggests either a lower level of *cdtA* and *cdtB* gene transcription in CD196 (about 30- to 60-fold) than that of iota toxin genes in *C.*

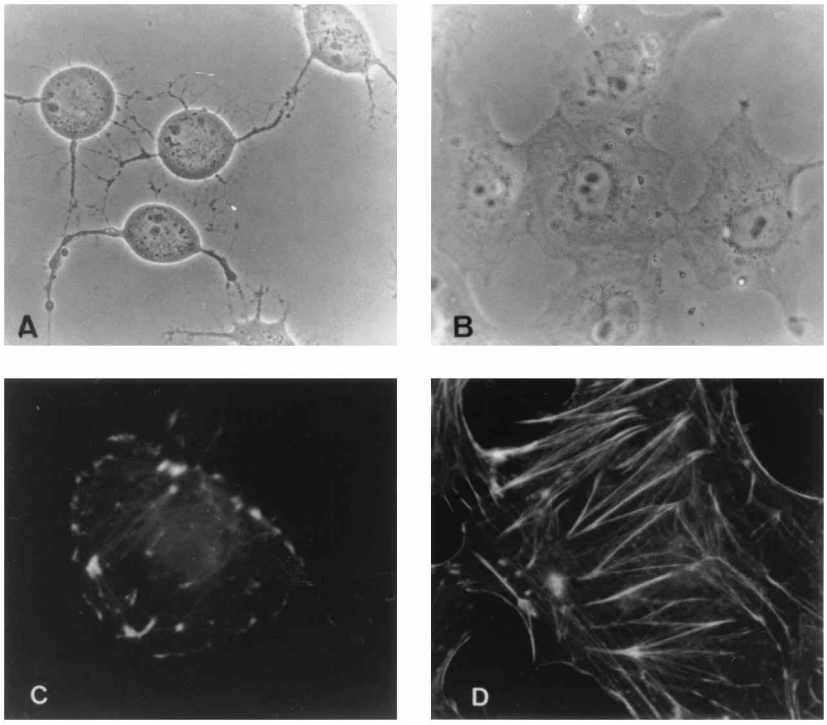

FIG. 4. CDTa and CDTb activity on Vero cells. Concentrated culture supernatant of CD196 (80 μg of total protein per ml) was treated with trypsin (200 μg/ml) for 20 min at room temperature, blocked with trypsin inhibitor (400 μg/ml), and exposed to Vero cells for 4 h. (A) Phase contrast of morphological changes induced by CDTa and CDTb; (B) neutralization with anti-Ib antibodies; (C and D) F actin staining of panels A and B, respectively. Control cells (not shown) are identical to those in panels B and D.

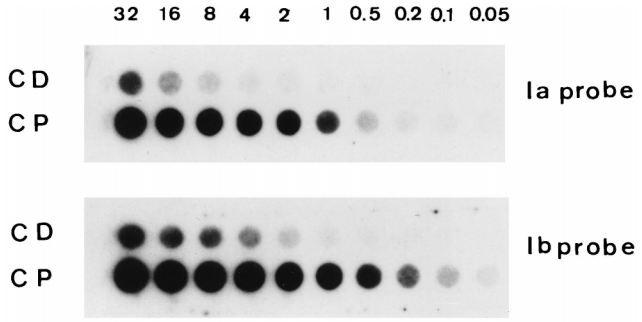

FIG. 5. Transcription analysis of the iota toxin, *cdtA*, and *cdtB* genes by RNA dot blotting in *C. perfringens* NCIB10748 (CP) and *C. difficile* CD196 (CD) with Ia and Ib probes. The total amount of RNA (in micrograms) applied in each well is indicated.

perfringens NCIB10748 or a different stability of mRNA between these strains. By Northern blot analysis, *cdtA* and *cdtB* probes hybridized to the same mRNA (approximately 5.8 kb [Fig. 6]), indicating that *cdtA* and *cdtB* are organized in an operon, as was found previously for *C. perfringens* iota toxin genes (14).

Distribution of the *cdtA* and *cdtB* genes among *C. difficile* clinical isolates. The presence of the *cdtA* and *cdtB* genes in 24 clinical isolates of *C. difficile* was tested by Southern blotting with *cdtA* and *cdtB* gene probes. Both *cdtA* and *cdtB* were identified in three strains (12.5%), and the *cdtB* gene alone was found in two additional strains. All the strains harboring *cdtA* and/or *cdtB* also produced ToxA and ToxB. The presence of CDTa and CDTb in culture supernatant was confirmed by Western blotting for only one strain (4%). This discrepancy between the DNA and protein analysis could be due to the presence of silent *cdtA* and *cdtB* genes, to *cdtA* and *cdtB* expression levels below the detection limit with anti-iota toxin antibodies instead of anti-CDT antibodies, or to CDTa and CDTb amino acid sequence variations such that the variants were not recognized by the anti-Ia and anti-Ib antibodies. These results indicate that the *cdtA* and *cdtB* genes not only are present in one atypical strain but also can be detected in certain clinical isolates.

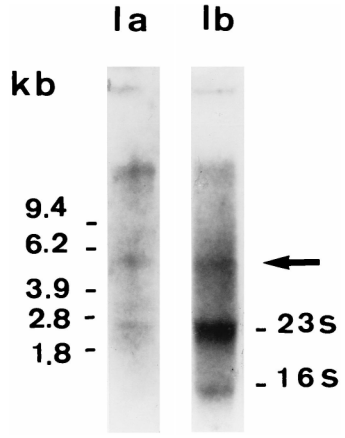

FIG. 6. Northern blot analysis of *C. difficile* CD196 RNA. Hybridization of ^{32}P-labeled Ia and Ib gene probes to a Northern blot of CD196 total RNA revealed a single mRNA transcript of approximately 5.8 kb (arrow).

DISCUSSION

In this study, we have shown that *C. difficile* CD196, which produces actin-specific ADP-ribosyltransferase, contains two genes (*cdtA* and *cdtB*) encoding a binary toxin homologous to the *C. perfringens* iota toxin (13). *cdtA* encodes the enzymatic component (CDTa) and *cdtB* encodes the binding component (CDTb), which have 81 and 84% amino acid sequence identity with the corresponding components of iota toxin (Ia and Ib, respectively). The *cdtA*, *cdtB*, and iota toxin genes present similar organizations. The enzymatic-component gene is localized in the 5′ part of the binding-component gene. The genes are transcribed in the same orientation and are separated by a short stretch of noncoding nucleotides (40 nucleotides in *C. perfringens* and 52 in *C. difficile*). The coding sequences of these genes in *C. difficile* and *C. perfringens* are very similar (80% identity), whereas the flanking noncoding sequences are less similar (40 to 61% identity). Possibly, the *cdtA*, *cdtB*, and iota toxin genes derive from a common ancestor, by interspecies gene transfer of a DNA fragment carrying the ancestral toxin genes. However, the DNA identity is greater between the binary toxin gene coding sequences than between their flanking regions, indicating a greater divergence of the noncoding segments during evolution. *iap* and *ibp* form an operon under the control of the promoter upstream of *iap* (14). The *cdtA* and *cdtB* genes probably also form an operon, since a long mRNA encompassing both genes has been identified. The sequence differences between the promoter regions in CD196 and *C. perfringens* NCIB10748 may explain the lower level of transcription of the *cdtA* and *cdtB* genes and consequently the lower level of expression of *cdtA* and *cdtB* than of the iota toxin gene. However, the smaller amount of *cdtA* and *cdtB* mRNA in CD196 than of iota mRNA in *C. perfringens* could also be due to different stabilities of mRNA in the strains.

We demonstrate that *C. difficile* CD196 produces a fully active iota-like toxin, which could be an additional virulence factor. The low levels of CDTa and CDTb expression in culture do not necessarily indicate that this toxin is produced in only small amounts in the digestive tract. Several binary-toxin-producing *Clostridium* organisms have been implicated in digestive diseases: *C. perfringens* E in animal enterotoxemia (4) and toxigenic *C. spiroforme* in rabbit enteritis (4) and in one case of colitis in a human (3). In addition, *C. botulinum* C2 toxin can induce necrotic and hemorrhagic lesions of the intestine and lungs (7). In this respect, it is noteworthy that CD196 was isolated from a patient with severe pseudomembranous colitis (17). Conceivably, CDTa and CDTb could induce intestinal lesions independently of ToxA and ToxB and/or produce synergistic effects with these toxins. Further, since investigation of 24 *C. difficile* clinical isolates showed that 12.5% of strains harbored the *cdtA* and *cdtB* genes and that one of them (4%) synthesized CDTa and CDTb, CDT is certainly not required for the virulence of *C. difficile*, but it is conceivable that the CDT-producing strains could correspond to a particular pathogenic *C. difficile* group.

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