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The Enzymatic Domain of *Clostridium difficile* Toxin A Is Located within Its N-Terminal Region

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***Clostridium difficile,* an anaerobic pathogen encountered in human enteric disease, produces two major virulence factors, toxins A and B, which are members of a clostridial family of large cytotoxins. These are glucosyltransferases, which use a UDP-sugar as co-substrate to glucosylate and inactivate small GTPases of the Rho or Ras families, culminating in cytotoxicity. Clinically, toxin A is perhaps the most important family member, because it causes major tissue damage in the course of disease, leading to a potentially lethal, pseudomembranous colitis. The location of the enzymatic domain of toxin A and mechanistic details of its action are not yet known, so we wished to localize this domain using gene deletion constructions from the full-length gene and by monitoring glucosylation activity of encoded protein products. Toxin A deletions were obtained by successively truncating the C-terminal coding region. These were transformed into *E. coli,* cell lysates were prepared and they were assayed for their ability to glucosylate Rho A protein, using an *in vitro* enzymatic assay. We report that the UDP-glucose binding site, the catalytic site for glucose transfer and the Rho A interaction site occur within the first 659 N-terminal amino acids of toxin A, i.e., less than 25% of the length of holotoxin A. Localization of the enzymatic domain of toxin A to these 659 N-terminal amino acids should greatly simplify studies on mechanistic details of this clinically important toxin.**

Clostridium difficile, frequently involved in antibiotic-induced diarrhea, produces two major virulence factors, toxin A, which has enterotoxic and cytotoxic activities, and toxin B, which has only cytotoxic activity (1-3). These toxins, along with α-toxin of *C. novyi* and both lethal and hemorrhagic toxins of *C. sordellii,* compose a clostridial family of large toxins (4). They show significant amino acid sequence similarity, possess glucosyltransferase activity, use a UDP-sugar co-substrate, and catalyze transfer of the glucose moiety to small GTPases of the Rho or Ras families, thus inactivating these signal transduction proteins (4,5). For example, toxin A monoglucosylates threonine 37 of Rho family members, but it is inactive against Ras proteins (6). Such covalent modification of Rho A results in actin cytoskeleton disassembly (7), as evidenced by cell rounding and eventually cytotoxicity (4-7).

The toxin A gene is 8.1 kbp, encoding a 2710 amino acid polypeptide of 308 kDa (8,9). Other smaller, more well-studied protein toxins, e.g., diphtheria toxin (10), are organized structurally and functionally into modular elements: {1} a binding domain for specific attachment to the host cell, prior to internalization; {2} a transfer domain for transport through the host cell membrane into the cytoplasm, prior to intoxication; and {3} an enzymatic domain, mediating cytoplasmic toxicity. But the extraordinarily large size of toxin A presents a dilemma for studies of structural organization and toxicity. Nevertheless, a modular domain organization was hypothesized for clostridial large toxins (4). Supporting this, a putative toxin A receptor binding domain was localized to its C-terminal repeat, amino acids (11); the *C. difficile* toxin B enzymatic domain was localized to its N-terminal 546 amino acids (12); and, the *C. sordellii* lethal toxin enzymatic domain was localized to its N-terminal 546 amino acids (13). We report here for the first time the localization of the toxin A enzymatic domain and its associated functions to the N-terminal 659 amino acids. These results further support the modular domain hypothesis for the clostridial family of large cytotoxins with the demonstration of a structurally and functionally indepen-

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Abbreviations: kbp, kilobase pairs; PCR, polymerase chain reaction; SDS, sodium dodecylsulfate; PAGE, polyacrylamide gel electrophoresis.

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dent, enzymatic domain within the full-length holotoxin A.

EXPERIMENTAL PROCEDURES

Materials, bacterial strains, plasmids, and chemicals. Native toxin A (gift of Dr. Rolfe, TTUHSC, Lubbock, TX) was dissolved (1 mg/ml in buffer: 1mM Tris-HCl, pH 7.5, 0.1mM EDTA). Rabbit polyclonal antiserum against toxoid A was a gift from Dr. Willis (Meridian Diagnostics, Inc., Cincinnati, OH). Mouse monoclonal antibodies were against full-length toxoid A[3] or Rho A (Santa Cruz Biotechnology, Santa Cruz, CA). *E. coli* strains, DH5αF'IQ (GIBCO BRL, Gaithersburg, MD), Epicurian Coli XL1-Blue (Stratagene, La Jolla, CA), BL21DE3 and NovaBlue (DE3) (Novagen, Inc., Madison, WI) were used throughout. Restriction enzymes, T4 kinase, T4 DNA ligase, Vent (exo^+) and (exo^-) DNA polymerases and Klenow were from New England BioLabs (Beverly, MA), USB (Cleveland, OH), or Promega Co. (Madison, WI). Plasmids, pIBI25 and pBR322, were used for subgenomic libraries to get clones A and C (14). T7 expression plasmid, pET20b(+) (Novagen, Madison, WI), was used for catalytic domain studies. Plasmid DNA was made by a boiling lysis method. DNA and RNA markers (GIBCO BRL, Gaithersburg, MD), synthetic oligos (Integrated DNA Technologies, Coralville, IA), nucleotides, [α-^{32}P]-ATP and [γ-^{32}P]-ATP (3000 Ci/mmol; New England Nuclear, Boston, MA), uridine diphosphate glucose, [glucose-1-^{3}H, 11.8 Ci/mmol], and chemicals (Sigma-Aldrich Chemical, St. Louis, MO; Baxter Scientific Products, McGaw Park, IL; Fisher Scientific, Pittsburgh, PA) were from indicated suppliers.

Reconstruction of the full-length toxin A gene. All molecular techniques were as described (15,16). Subgenomic libraries were made from restriction enzyme digested, sucrose gradient fractionated DNA (14). Clone A was a 12.6 kbp EcoR V DNA cloned in the same site of pIBI25 (14). Clone C was an 11.8 kbp Nde I and Cla I fragment cloned into the same sites of pBR322. Toxin 5′ and 3′-end fragments were made by PCR and cloned into pET20b. The PCR had 20 mM Tris-HCl, pH 8.8, 2 mM $MgSO_4$, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 50 μM each dNTP, 100 pmoles primer, 1ng DNA template, 1 U Vent (exo^-) and 0.01 U Vent (exo^+) DNA polymerase in 50 μl. Samples were amplified 30 cycles (30 s at 94°, 1 min at 52° and 2 min at 72°), run in 0.8% agarose gels, electroeluted and DNA amounts estimated by comparison with ethidium bromide stained standards. Central toxin A gene fragments, obtained from clones A and C, were inserted sequentially into the pET20b constructs. Plasmids were transfected into *E. coli* strain, DH5αF'IQ, by electroporation. Electrocompetent cells were prepared in 500 ml Luria broth grown to an optical density of 0.8 at 600 nm, collected by centrifugation, washed in 50 ml Luria broth and resuspended in 2 ml of 10% glycerol and 90% distilled water. Desired clones were detected by Grunstein-Hogness hybridization, using probes labeled with [γ^{32}P]-ATP or [α^{32}P]-ATP. Construct authenticity was confirmed by restriction enzyme mapping and DNA sequencing.

Construction of toxin A gene deletion mutants. Truncated mutants were made from the full-length toxin A gene, using the restriction enzymes, Hind III, EcoR I, and Swa I, to obtain a nested deletion series. Mutants were characterized by restriction enzyme digestion and sequenced through deletion site junctions by DNA sequence analysis at the Core Facility in the Department of Chemistry and Biochemistry, Texas Tech University, with synthetic oligonucleotide primers and terminator-labeled with fluorescent dye, using the ABI DNA sequencer. Cycle sequencing was also performed by us, using Vent (exo^-) DNA polymerase with oligonucleotide primers, end-labeled with [γ-^{32}P]ATP, and reactions were run on denaturing 5% polyacrylamide gels, followed by autoradiography.

[3] Faust, unpublished data.

Recombinant toxin A cell lysates and Rho A protein. Cells were grown to an optical density of 0.7 at 600 nm and 30° in 50 ml of Luria broth containing ampicillin (60 μg/ml). T7 expression of recombinant toxins was induced by adding IPTG to 0.6 mM, and incubation continued 2 h at 30°. All steps were at 4° to preserve a labile toxin activity. Cells were centrifuged at 2,000 × g for 10 minutes (Beckman J-6B), resuspended in 1 ml of 50 mM Tris-HCl, pH 8.0, with 6mM phenylmethyl-sulfonyl fluoride (PMSF) and 2 mg/ml of lysozyme, and briefly sonicated on ice. After every 4 cycles (8 s) the sample was cooled for 10 s, and this was repeated 5 times. Cell debris was removed by centrifugation at 13,000 × g for 12 min (Beckman J2-MC, JA-18.1 rotor). Protein concentration was measured by Bradford assay, and glucosylation activity in the cell lysates was determined.

Recombinant Rho A proteins were obtained from *E. coli* strain, JM101, transformed with the cloned construct, pGEX-2T/Rho A (a gift of Dr. A. Hall, MRC Lab, London, UK) (17). Rho A was cloned into pGEX-2T, which is designed for IPTG inducible expression in fusion with *Schistosoma japonicum* glutathione S-transferase (18). Rho protein was released from the 26 kDa glutathione S-transferase by thrombin, and was subsequently recovered with glutathione agarose affinity chromatography (17).

Toxin A enzymatic activity assays. An *in vitro* glucosylation, microassay of Rho A protein was developed from previously reported procedures (19,20). Briefly, 200 nCi [^{3}H]-labeled UDP-glucose and 1 μg recombinant Rho protein were used in 20 μl reactions. Ten μl of recombinant, *E. coli* cell lysate, containing 30 μg of protein, were dialyzed against 50 mM Tris-HCl, pH 7.5, for 30 minutes on ice, using a floating Millipore microfilter (pore size, 0.025 μm), before its use in the 20 μl glucosylation reaction. Reactions were run at 30° for 2 h and stopped with 5 μl of sample loading buffer (0.16 M Tris-HCl pH 6.8, 2.5% SDS, 37.5% glycerol, 150 mM dithiothreitol and 0.05% bromophenol blue dye). Samples were run on SDS 15% PAGE, dried on Whatman 3MM paper and fluorographs exposed to film. Alternatively, proteins were transferred to Immobilon P (Millipore, Bedford, MA) membranes, and fluorographed using the Kodak BioMax TranScreen and film system with an LE intensifying screen. A standard curve for Rho A protein glucosylated by native toxin A was established under the conditions described above. Enzymatic activity was likewise measured in the lysates of the deletion mutants. Both the optical density from densitometry and the radioactivity from scintillation counting were obtained, providing a relative value of glucosylation activity in the deletion mutant samples.

Immunodot blot and Western blot analyses. *E. coli* cell lysates were blotted to nitrocellulose or Immobilon P membranes, using a dotblot apparatus, and air-dried briefly. Serial two-fold dilutions were in a 200 μl volume of TBS buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl), and incubations and washes were at room temperature. Membranes were incubated with primary antibody against toxin A (1:20,000 dilution of mouse monoclonal antibody #12 or rabbit polyclonal antiserum in 10 ml of TBST buffer: TBS with 0.05% Tween-20) for 30 min, washed three times 10 min each with TBST, blocked 2 h in 3% gelatin in 10 ml of TBST, incubated 30 min with horseradish peroxidase conjugated secondary antibody (1:20,000 of goat anti-mouse IgG or goat anti-rabbit IgG) in 1% gelatin in TBST and unbound antibodies removed by three 10 min TBST washes. Bound antibodies were detected using the Phototope-Horseradish Peroxidase Western blot detection kit (New England Biolabs, Ltd., Beverly, MA) or by incubation with SuperSignal Chemiluminescent Substrate with luminol for 5 min (Pierce, Rockford, IL). Western blots were obtained from cell lysates run for 1 h at 200 V on SDS 15% PAGE with electrophoretic transfer for 1 h at 100 V, and immunoblotted the same as for the dot blots. Chemiluminescent images were captured on X-ray film and quantified using the Visage 2000 BioImage System (Genomic Solutions Inc., Ann Arbor, MI).

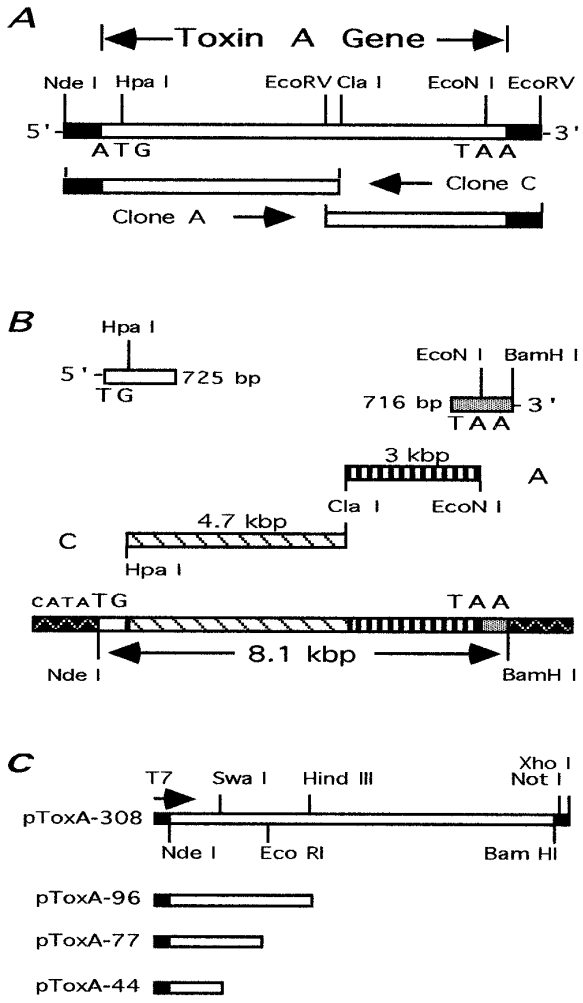

FIG. 1. Scheme for construction of the toxin A gene and its deletion mutants. Structures in (A) and (B) are not drawn to scale. (A) A and C from *C. difficile* genomic sublibraries (14). (B) 8.1 kbp toxin A gene constructed in pET20b. About 100 and 300 of the 725 and 716 bp products are in the gene. Boxes, flanking the 8.1 kbp DNA, are from pET20b and contain Nde I and BamH I sites. Unique sites in (A) and (B): Hpa I, Cla I, EcoN I, Nde I and BamH I. (C) T7 promoter in pToxA-308 and deletion sites. Toxin A gene sites are proportionately spaced to display comparable deletion mutant lengths, relative to the gene. BamH I, Not I and Xho I in pET20b are close to each other and the toxin gene, but are not proportionately spaced. Unique sites are Nde I, BamH I, Not I and Xho I. Others occur multiply, but only relevant ones are shown. Short vector peptides occur in fusion to the C-terminus of toxin A: 21 amino acids for pToxA-308, 11 for pToxA-96, 11 for pToxA-77 and 7 for pToxA-44.

RESULTS AND DISCUSSION

Toxin A of *C. difficile* plays a major role in the etiology of antibiotic-associated, human enteric disease. This single, large polypeptide of 308 kDa is encoded by a gene that, together with the toxin B gene, may be expressed as part of a multigene operon (21,22). However, because of the inability to study the genetics of toxin A in *C. difficile* and the large size of toxin A, progress has been difficult in obtaining details of toxin A structural organization and its mechanism of toxicity. Also, genes of Gram-positive organisms, such as *C. difficile*, are frequently expressed poorly, if at all, in Gram-negative organisms, making studies in *E. coli* difficult. Nevertheless, because of our previous success in *E. coli* (14), this gene deletion and expression study was done to localize the toxin A glucosylation domain, so as to gain more insight eventually into its enzymatic mechanism of cytotoxicity.

Construction of a full-length toxin A gene, pToxA-308, for expression in E. coli. *C. difficile* genomic sublibraries were made (14) to get clones A and C (Fig. 1A) to construct a toxin A gene in the *E. coli* expression vector, pET20b, as follows. The 725 bp gene 5′-end was made by PCR with primers, A1 and A2 (Fig. 1B and Table I). Primer A1, was complementary to the toxin A antisense strand, so as to reconstruct an authentic translation initiation codon, ATG, into the vector. Opposed primer, A2, was complementary to the sense strand 725 bp downstream. Likewise, the 716 bp toxin A 3′-end used primer pair, A3 and A4, where A4 has a BamH I recognition site (Fig. 1B and Table I).

The cloning strategy (Fig. 1B) put the 725 bp PCR 5′-end product into pET20b, prepared by Nde I digestion and blunt-ended by Klenow fragment, generating pET5′. Next, the 716 bp 3′-end product (Fig. 1B), digested by BamH I, was cloned into pET5′, first treated with Nco I, blunt-ended by Klenow fragment and digested by BamH I for compatible sites, generating pET5′/3′. A full-length toxin A gene was prepared by adding two central portions, obtained from subgenomic library clones, A and C. The 3 kbp piece, from an EcoR V and EcoN I digest of clone A, was inserted into the same sites in pET5′/3′ (Fig. 1B), yielding pET5′/A/3′ with a unique Cla I site. The 4.7 kbp portion of the gene, from a Cla I and Hpa I digest of clone C, was inserted into the compatible sites of pET5′/A/3′ (Fig. 1B), generating a toxin A gene, pToxA-308 (pET5′/C/A/3′). This was characterized by numerous restriction

TABLE I

Oligonucleotides Used

Oligo	DNA sequence (5′ → 3′)	Size/Polarity
A1	TGTCTTTAATATCTAAAGAAGAG	23-mer (+)
A2	TAGCCCTGATATCTATCCCAT	21-mer (−)
A3	CACTGCTGTTGCAGTTACTGG	21-mer (+)
A4	CGGGATCCCCATATATCCCAGGGGCTTT	28-mer (−)
P1	TAATACGACTCACTATA	17-mer (+)
P2	CTAGTTATTGCTCAGCGG	18-mer (−)

Note. Polarity relates to toxin A sense (+) translational reading frame. A1 to A4 are from toxin A gene; P1 and P2 are from pET20b, flanking the toxin A gene.

enzyme digestions.[4] It was also cut by Nde I and BamH I, releasing an 8.1 kbp full-length, toxin gene, thus confirming at the 5′-end the authentic translation initiation codon, ATG, in the Nde I site, CATATG, while also verifying the 3′-end of the construct (Fig. 1B). Finally, DNA sequencing was done through the 0.7 kbp 5′ and 3′-ends obtained by PCR, using primers, P1 and P2 (Table I), thus verifying coding sequences through the Hpa I and EcoN I sites of the respective PCR products.[5]

Construction of a series of toxin A gene truncation mutants. The hypothesis examined here is that the N-terminal portion of toxin A contains the enzymatic domain. Therefore, nested deletions were made to find the shortest length encoding glucosylation activity. Three deletion mutants were prepared from the full-length, gene construct, pToxA-308 (Fig. 1C). DNA was digested by Hind III and Not I, blunt-ended by Klenow and circularized giving construct, pToxA-96. This has 2481 bp of the 5′-end, encoding a 96 kDa toxin A polypeptide, predicted to have 827 amino acids of the N-terminus with termination at codon 839. Digestion of pToxA-308 by EcoR I and Not I, processed as above, generated another deletion construct, pToxA-77, which has 1977 bp of the 5′-end, encoding a 77 kDa toxin A polypeptide, predicted to have 659 N-terminal amino acids with termination at codon 671. Finally, toxin A deletion construct, pToxA-44, was generated by Xho I digestion, filled in with A, C and G by Klenow fragment, Swa I digested and the blunt ends ligated. This has 1094 bp of the 5′-end fused to the vector, encoding a 44 kDa toxin A polypeptide, predicted to have 364 N-terminal amino acids, since ligation creates a proximal termination codon at position 372. All deletion mutant DNA sequences were verified through their 3′-end coding sequences,[6] using primer, P2 (Table I).

Production of toxin A proteins in E. coli. In order to localize a glucosylation domain in the truncated toxin A peptides, all the constructs were expressed in the *E. coli* strain, BL21DE3, which contains an inducible, T7 RNA polymerase gene. The amount of toxin peptides in cell lysates was determined by immunodot blot with a mouse monoclonal antibody recognizing toxin A (Fig. 2A), together with total protein. Results indicated similar amounts of toxin A products exist in the cell lysates of all deletion mutants tested. In contrast, cell lysates with only pET20b did not react with antibody.

A monoclonal antibody was used to find clones producing similar amounts of toxin A products to facilitate comparison. A polyclonal antiserum was considered less satisfactory, since it would contain a heterogeneous antibody population that would disproportionately recognize an indeterminate number of epitopes, not uniformly present on the different toxin A product lengths. This problem would not occur with a monoclonal antibody, if it only recognized a non-repeating epitope present on the products of all clones. Extensive repeats do occur in the toxin A C-terminus (11), but computer analysis of our deletion clones indicated they contained no repeated elements.[7] Thus, monoclonal antibody #12 must recognize a unique epitope within the N-terminus of toxin. Therefore, these deletion clones produced comparable amounts of toxin A products, as defined by this unique epitope.

In contrast, a polyclonal, monospecific antiserum was used in western blots for a perspective of all toxin A-related products. All clones, except pToxA-308, produced detectable amounts of predicted, size products (Fig. 2B). The largest protein detected in the pToxA-96 lysate is 96 kDa; in the pToxA-77 lysate, it is 77 kDa; and in the pToxA-44 lysate, it is 44 kDa, consistent

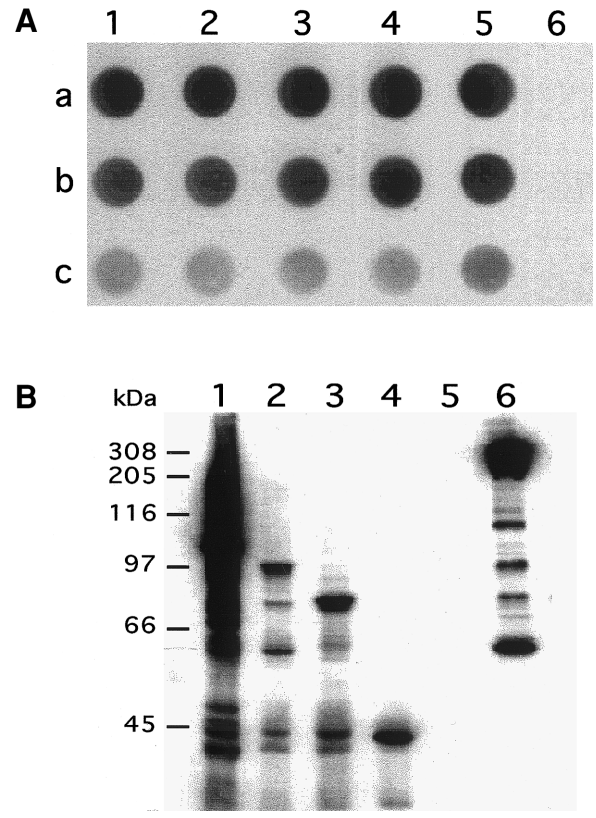

FIG. 2. Production of toxin A peptides in *E. coli*. (A) Immunodot blot assayed with monoclonal antibody #12: lane 1, 2 μg, 1 μg, and 0.5 μg of native toxin A on a, b and c; lanes 2 to 5, *E. coli* lysates from pToxA-308, pToxA-96, pToxA-77 and pToxA-44; lane 6, lysate from pET20b. Protein on dots a, b and c: 12 μg, 6 μg and 3 μg for lanes 2 to 6. (B) Western: lanes 1 to 4 contain 3 μg protein from pToxA-308, pToxA-97, pToxA-77 and pToxA-44; lane 5, 3 μg lysate from pET20b; lane 6, 1 μg, native toxin A.

[4] Ye and Faust, unpublished data.
[5] Faust, unpublished data.
[6] Ye and Faust, unpublished data.
[7] Faust, unpublished data.

with sizes predicted for the deletion constructs. However, the largest product from pToxA-308 was only about 180 kDa. Similar amounts of small, toxin A products were found in lysates from toxin A gene constructs. These smaller sized products might be due to proteolysis. Alternatively, major G-C differences between the *E. coli* (50%) genome and toxin A gene (26.9%) of *C. difficile* (8,9,23) may result in translational pauses. This would be expected from the strong bias for AT-rich codons used more frequently in the toxin genes of *C. difficile* (8,9,23), compared to those codons frequently used in *E. coli* (24,25). The amount of expected, size product could not be increased further, despite many attempts with various protease inhibitors or altered cell growth conditions, suggesting that translational pauses may be principally responsible for the smaller products observed.

Localization of the enzymatic domain of toxin A within its N-terminus. Glucosylation activity in each cell lysate was determined by the covalent transfer of [^{3}H]-glucose from the co-substrate, UDP-glucose, to Rho A protein (Fig. 3). The amount of [^{3}H]-glucose in each Rho A protein band labeled by the lysate of each deletion mutant was measured (Table II). The results show that pToxA-96 and pToxA-77 lysates contain similar levels of activity, while the pToxA-308 lysate has only about half of this activity, and the pToxA-44 lysate has none. Thus, pToxA-44 product has no active glucosylation domain, since both immunodot and Western blots revealed similar amounts of toxin products for all deletion constructs. These results also suggest that toxin products smaller than 45 kDa are likely inactive, regardless of the lysate from which they originate.

In conclusion, an enzymatic domain for binding UDP-glucose, for catalytically transferring glucose to Rho A and for recognizing the interaction interface of Rho A resides in the first N-terminal 659 amino acids of toxin A, i.e., about 24% of its 2710 amino acids. This is comparable to the N-terminal fraction from *C. difficile* toxin B (12) or from *C. sordellii* lethal toxin (13) needed for glucosylation, i.e., only 546 amino acids of these highly related holotoxins are needed, or 23% of their lengths. These results provide additional evidence in support of the domain hypothesis suggested for the clostridial family of large cytotoxins (4). This work also indicates that any subsequent mechanism studies done to identify essential amino acids within the enzymatic domain of toxin A would greatly benefit from use of the more simple, pToxA-77 construct characterized here. Since pToxA-77 encodes only the N-terminal 659 amino acids of the full-length, 2710 of holotoxin A, this construct eliminates 75% of toxin A from any site-directed mutagenesis to identify essential amino acids or to study other structural features within this domain essential for glucosylation.

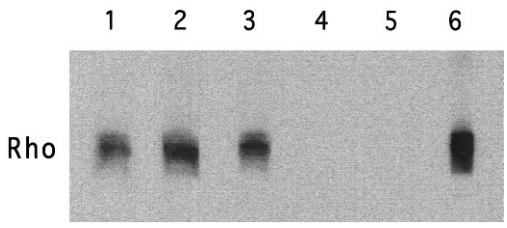

FIG. 3. Determination of toxin A-mediated, enzymatic activity by glucosylation of Rho A protein. Lanes 1 to 4, Rho A treated with lysates from pToxA-308, pToxA-96, pToxA-77 or pToxA-44, respectively; lane 5, Rho A treated with pET20b lysate; lane 6, Rho A treated with native toxin A.

TABLE II

Relative Glucosylation Activities in the Lysates of the Deletion Mutants Used in This Study

Deletion mutant	[^{3}H]-Rho A, pCi/μg
pToxA-308	270
pToxA-96	580
pToxA-77	560
pToxA-44	0

Note. Activity is expressed as [^{3}H]-labeled Rho A protein per 20μl reaction minus pET20b bkg.

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