Role of Aromatic Amino Acids in Receptor Binding Activity and Subunit Assembly of the Cytolethal Distending Toxin of *Aggregatibacter actinomycetemcomitans*[▽]

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The periodontal pathogen *Aggregatibacter actinomycetemcomitans* produces a cytolethal distending toxin (Cdt) that inhibits the proliferation of oral epithelial cells. Structural models suggest that the CdtA and CdtC subunits of the Cdt heterotrimer form two putative lectin domains with a central groove. A region of CdtA rich in heterocyclic amino acids (aromatic patch) appears to play an important role in receptor recognition. In this study site-specific mutagenesis was used to assess the contributions of aromatic amino acids (tyrosine and phenylalanine) to receptor binding and CdtA-CdtC assembly. Predominant surface-exposed aromatic residues that are adjacent to the aromatic patch region in CdtA or are near the groove located at the junction of CdtA and CdtC were studied. Separately replacing residues Y105, Y140, Y188, and Y189 with alanine in CdtA resulted in differential effects on binding related to residue position within the aromatic region. The data indicate that an extensive receptor binding domain extends from the groove across the entire face of CdtA that is oriented 180° from the CdtB subunit. Replacement of residue Y105 in CdtA and residues Y61 and F141 in CdtC, which are located in or at the periphery of the groove, inhibited toxin assembly. Taken together, these results, along with the lack of an aromatic amino acid-rich region in CdtC similar to that in CdtA, suggest that binding of the heterotoxin to its cell surface receptor is mediated predominantly by the CdtA subunit. These findings are important for developing strategies designed to block the activity of this prominent virulence factor.

One of the recurring themes in secreted bacterial protein toxins that act intracellularly is a structure composed of heterogeneous subunits or polypeptides, each of which has a distinct role in toxin activity. Classical examples include cholera (22), diphtheria (6), and anthrax (3) toxins and *Pseudomonas* exotoxin (11). These are known as A-B-type toxins because they contain at least one subunit or polypeptide that recognizes a specific receptor on the cell surface and one subunit or polypeptide that enters the cell to gain access to the target site. The cytolethal distending toxin (Cdt) of the oral bacterium *Aggregatibacter* (formerly *Actinobacillus*) *actinomycetemcomitans*, like the Cdt found in other pathogenic species, is a member of this A-B class of cytotoxins (15). However, Cdt has a unique variation of the typical A-B paradigm. Biologically active Cdt from all toxin-producing species examined to date, with the exception of that found in *Salmonella enterica* serovar Typhi (9), is a trimer containing two heterologous subunits, CdtA and CdtC, that are predicted to interact with the cell surface receptor. The third subunit, CdtB, is the cytotoxic component of the heterotrimer complex and has to enter cells to elicit toxic effects.

The crystal structures of the Cdt from *A. actinomycetemcomitans* (23) and the phylogenetically related organism *Haemophilus ducreyi* (18) include a pronounced groove between

the CdtA and CdtC subunits in the heterotoxin complex. In addition, a region enriched in surface-exposed aromatic (tyrosine) and heterocyclic (tryptophan) amino acids, termed the aromatic patch, was identified in the *H. ducreyi* CdtA (18). A mutated CdtA protein containing the substitutions W91G, W98G, W100G, and Y102A formed a heterotrimer that failed to bind to HeLa cells (19). In the same study, a Cdt groove mutant formed by reconstituting proteins from two double-amino-acid substitution mutants, CdtA(P103A, Y105A) and CdtC(R43K, Q49A), exhibited diminished binding to cells. Collectively, these data support the hypothesis that the aromatic patch in CdtA and the groove formed between CdtA and CdtC are involved in the binding of the toxin to target cells.

The individual CdtA and, to a lesser extent, CdtC subunit proteins bind to cells in culture (2, 12, 13, 16) and in an enzyme-linked immunosorbent assay with cells (CELISA) (4, 13). The cell surface receptor for the toxin has not been identified. However, it has been reported that both CdtA and CdtC recognize N-linked fucose-containing glycoproteins (16) or gangliosides, such as GM1 and GM3 (17), in vitro. In other studies it has been suggested that CdtC may aid the entry of CdtB into the cell (1, 7, 8). At present, it is not clear how CdtA and CdtC interact with the cell receptor and with each other and possibly carry out other functions related to cytotoxicity.

We have been using a site-directed mutagenesis approach to learn more about the specific interactions of the CdtA and CdtC subunits (4, 5). The goal of this study was to obtain specific amino acid substitution mutants with mutations in the *cdtA* and *cdtC* genes of *A. actinomycetemcomitans* Y4 and to

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TABLE 1. Site-directed mutagenesis of surface-exposed aromatic residues in CdtA and CdtC

Plasmid	Amino acid change(s)	Primer	Sequence ^a
pMUT105cdtA	Y105A	CdtA-Y105A-F CdtA-Y105A-R	5'-GGGCTTATCCCAATATAGCTTCGACGACTTTGG-3' 5'-CCAAAGTCCTGCGAAGCTATATTGGGATAAGCCC-3'
pMUT140cdtA	Y140A	CdtA-Y140A-F CdtA-Y140A-R	5'-GGTACATGTATTGAAGCTGCCGGTAATGGTTTAATTCAT-3' 5'-CATGAATTAACCATTAACCGGCAGCTTCAATACATGTACC-3'
pMUT188cdtA	Y188A	CdtA-Y188A-F CdtA-Y188A-R	5'-TAATCCTGTAAGTCCAACAGCTTATTCAACAGTTAC-3' 5'-GTAAGTGTGAATAAGCTGTTGGACTTACAGGATTA-3'
pMUT189cdtA	Y189A	CdtA-Y189A-F CdtA-Y189A-R	5'-CCTGTAAGTCCAACATATGCTTCAACAGTTACATTATC-3' 5'-GATAATGTAAGTGTGAAGCATATGTTGGACCTACAGG-3'
pMUT56cdtC	Y56A	CdtC-Y56A-F CdtC-Y56A-R	5'-CCAATTAATAATGACCATGCTGATTACATAATTATTTAAG-3' 5'-CTTAAATAATTATGTGAATCAGCATGGTCATTTTTAATTGG-3'
pMUT61cdtC	Y61A	CdtC-Y61A-F CdtC-Y61A-R	5'-GACCATTATGATTACATAATGCTTTAAGTACACATTGGG-3' 5'-CCCAATGTGTACTTAAAGCATTATGTGAATCATAATGGTC-3'
pMUT97cdtC	F97A	CdtC-F97A-F CdtC-F97A-R	5'-TAAAGTGGTTCGGTGCAGCAAAATGTGCTGCTTTCCAGGC-3' 5'-GCCTGGGAAAGCAGCACATTTTGCTGCACCGACACTTTA-3'
pMUT99cdtC	F99A	CdtC-F99A-F CdtC-F99A-R	5'-TCGGTGCAGCAAAATGTTTTGCTGCCCGAGGCGAAGG-3' 5'-CCTTCGCCTGGGGCAGCAAAACATTTTGCTGCACCGA-3'
pMUT97/99cdtC	F97A, F99A	CdtC-F97,99A-F CdtC-F97,99A-R	5'-GCAGCAAAATGTGCTGCTGCCCGAGGCGAAGGC-3' 5'-GCCTTCGCCTGGGGCAGCAGCACATTTTGCTGC-3'
pMUT115cdtC	F115A	CdtC-F115A-F CdtC-F115A-R	5'-TGATCATACTGTGGCTAACCTTATTCCAAC-3' 5'-GTTGGAATAAGGTTAGCCACAGTATGATCA-3'
pMUT134cdtC	F134A	CdtC-F134A-R CdtC-F134A-F	5'-CATGGCTTGTTCATACAAGCTCCTAATAGGGCATCTTTG-3' 5'-CAAAGATGCCCTATTAGGAGCTTGTATGACAAGCCATG-3'
pMUT141cdtC	F141A	CdtC-F141A-F CdtC-F141A-R	5'-CCATGACGCTGATGATTTGAGGCTTGAACC-3' 5'-GGTTCAAGCCTCAAATCATCAGCGTCATGG-3'
pMUT170cdtC	F170A	CdtC-F170A-F CdtC-F170A-R	5'-TGGGGAATATTACCTCCTGCTGGGC-3' 5'-GCCCAGCAGGAGGTAATATTCCCCA-3'

^a Underlined bases are the alanine codons.

use the gene products to further characterize the binding properties of the two subunits. A number of studies showing that binding of the Cdt subunits could be observed in vitro indicated that enzyme-linked immunosorbent assays (ELISA) could facilitate evaluation of the effects of mutations on the binding properties of CdtA and CdtC. Since aromatic amino acids contain a large reactive benzenoid ring that is prominently displayed when these residues are located at the surface of a protein, substitutions were made for surface-exposed aromatic residues that (i) border the aromatic patch identified in *H. ducreyi* CdtA and (ii) are in or at the periphery of the groove that is formed by the CdtA-CdtC heterodimer. The effects of these mutations on the binding properties of CdtA and CdtC are discussed below.

MATERIALS AND METHODS

Site-directed mutagenesis of *cdtA* and *cdtC*. Prominent surface-exposed aromatic amino acids in CdtA-His₆ and CdtC-His₆ were replaced, in individual mutants, by site-directed mutagenesis. Synthetic oligonucleotide primer pairs (Integrated DNA Technologies, Inc., Coralville, IA) were used to change each tyrosine or phenylalanine codon to an alanine codon (Table 1). Mutated DNA strands were made using PfuUltra DNA polymerase in PCR (Stratagene, La

Jolla, CA). Plasmid pJDA9 and pJDC2 DNA preparations were used as PCR templates for wild-type *cdtA* and wild-type *cdtC* mutagenesis, respectively, as described previously (4). Methylated parental DNA strands were digested with DpnI (New England Biolabs, Beverly, MA), and the remaining mutated DNA was transformed into *Escherichia coli* TOP10 [F⁻ *mcrAΔ(mrr-hsdRMS-mcrBC) φ80lacZΔM15ΔlacX74 recA1 araD139Δ(ara-leu)7697 galU galK rpsL(Str^r) endA1 nupG*] chemically competent cells (Invitrogen, Carlsbad, CA). Nucleotide changes were confirmed by sequencing plasmid insert DNA from a single transformant. Automated cycle sequencing reactions were conducted by the Genetics Core Facility at the University of Pennsylvania using an Applied Biosystems 377 sequencer with dye primer chemistry. Plasmid DNA containing a confirmed sequence was purified with a Wizard Plus Miniprep DNA purification system kit (Promega Corp., Madison, WI) and transformed into *E. coli* BL21(DE3) [F⁻ *ompT hsdS_B(r_B⁻ m_B⁻) gal dcm* (DE3)] competent cells (Novagen-EMD Biosciences, San Diego, CA) to express the mutated gene and for isolation of the gene product.

Expression of the mutated genes was assessed by examining whole bacterial lysates on Western blots using anti-His₆ Tag monoclonal antibody (Novagen-EMD Biosciences) and anti-mouse immunoglobulin G (IgG)-horseradish peroxidase conjugate (Amersham Pharmacia Biotech, Piscataway, NJ), both at a 1:3,000 dilution. Purified wild-type CdtA-His₆ and CdtC-His₆ proteins and prestained molecular weight standards (Bio-Rad Laboratories, Hercules, CA) were used as markers. Whole bacterial lysates from *E. coli* BL21(DE3)(pJDA9) and *E. coli* BL21(DE3)(pJDC2) were used as positive controls. Lysate from *E. coli* BL21(DE3)(pET15b) served as a negative control.

Polyclonal CdtB and CdtC antisera. Subunit-protein-specific antisera for CdtB-His₆ and CdtC-His₆, used in some experiments, were made in rabbits (Cocalico Biologicals, Inc., Reamstown, PA). IgG fractions were purified using a Montage antibody purification kit (Millipore, Billerica, MA). IgG titers were obtained by ELISA using purified CdtB-His₆ and CdtC-His₆ as antigens. Cross-reactivity with all three Cdt proteins was assessed by Western blotting. Bound IgG was detected with a 1:3,000 dilution of donkey anti-rabbit IgG-horseradish peroxidase conjugate (Amersham Pharmacia Biotech).

Isolation of recombinant wild-type and mutant subunit gene products and heterotoxin reconstitution. Recombinant clones *E. coli* BL21(DE3)(pJDA9), *E. coli* BL21(DE3)(pJDB7), and *E. coli* BL21(DE3) (pJDC2) were used to prepare the wild-type CdtA-His₆, CdtB-His₆, and CdtC-His₆ proteins, respectively. The proteins were obtained from isopropyl- β -D-thiogalactopyranoside (IPTG)-induced cultures by affinity chromatography on nickel-iminodiacetic acid columns (Novagen-EMD Biosciences) as described previously (14). The same procedure was used to obtain the mutated CdtA-His₆ and CdtC-His₆ subunit proteins. The average yields were 40 to 50 μ g of protein/ml of culture. The final protein preparations were dialyzed to remove urea while promoting protein refolding, passed through 45- μ m filters, and quantified with a Micro BCA protein assay kit (Pierce, Rockville, IL) as described previously (4). Purity was assessed by analysis on 10 to 20% sodium dodecyl sulfate-polyacrylamide gels stained with Coomassie brilliant blue. Aliquots of the quantified protein samples were stored at -70°C in a buffer containing 10 mM Tris-HCl (pH 7), 100 mM NaCl, 5 mM MgCl₂, and 5 mM imidazole.

Wild-type and mutant heterotoxins were reconstituted *in vitro* by mixing equimolar concentrations of the appropriate subunit proteins in a buffer containing 10 mM Tris-HCl (pH 7), 100 mM NaCl, and 5 mM MgCl₂. The mixtures were incubated for 1 h at 4°C and used immediately.

Receptor binding assays. A whole-cell CELISA, which was characterized and standardized previously (4), was used to measure direct binding of the mutated CdtA-His₆ and CdtC-His₆ subunit proteins to the native receptor. Briefly, 96-well microtiter plates were seeded with 1.5×10^4 Chinese hamster ovary (CHO-K1) cells/well in Ham's F-12 medium (Invitrogen-GIBCO, Carlsbad, CA) containing 5% fetal calf serum. The plates were incubated for 48 h at 37°C in an atmosphere containing 5% CO₂ to allow the cells to attach and become confluent. The plates were then washed with phosphate-buffered saline (PBS) containing 1 mM MgCl₂ and 1 mM CaCl₂, and the cells were fixed with 10% formalin for 15 min at room temperature. One microgram of each affinity-purified mutated protein (saturating concentration) in PBS containing 3% bovine serum albumin was added to triplicate wells. Other sets of triplicate wells received either no protein (background control) or 1 μ g of wild-type CdtA-His₆ or CdtC-His₆ (positive controls). Subunit protein concentrations were based on the results of saturation binding kinetics determined previously (4). The plates were incubated for 1 h at room temperature, washed, and fixed with a mixture of 2% formaldehyde and 2% glutaraldehyde. After extensive washing with PBS-0.1% Tween 20, bound protein was detected with a 1:3,000 dilution of anti-His₆ Tag monoclonal antibody, a 1:3,000 dilution of anti-mouse IgG-horseradish peroxidase conjugate, and 100 μ l/well of the horseradish peroxidase substrate ABTS-100 (Rockland, Gilbertsville, PA). The plates were read at a wavelength of 405 nm with a Synergy 2 microplate reader (BioTek Instruments, Inc., Winooski, VT). These experiments were repeated a minimum of three times.

The CELISA was also used to assess the binding of the mutated CdtA-His₆ subunit proteins when they were assembled as a heterodimer and as a heterotrimer. Wild-type or mutated CdtA (12 μ g) was incubated in 300 μ l refolding buffer for 1 h at 4°C alone or with equimolar concentrations of CdtC-His₆ or CdtC-His₆ plus CdtB-His₆. The preparations (100 μ l) were added to triplicate wells containing 1.5×10^4 CHO cells, and the plates were treated as described above. Absorbance ratios for the heterodimers and heterotrimers were calculated relative to the binding of the wild-type and mutated CdtA proteins, as in the thyroglobulin ELISA used for the subunit binding assay (see below) (4).

To assess ligand binding of the individual CdtA-His₆ and CdtC-His₆ substitution proteins, an ELISA based on immobilization of the fucose-containing glycoprotein thyroglobulin was used. This assay was thoroughly tested and standardized previously (4). Briefly, 96-well microtiter plates were precoated overnight with 75 μ g/well of thyroglobulin (Sigma-Aldrich, St. Louis, MO). The plates were washed with PBS-0.1% Tween 20. Ten micrograms (saturating concentration [4]) of each affinity-purified CdtA-His₆ or CdtC-His₆ mutated protein was added to triplicate wells. Other sets of triplicate wells received either no protein or 10 μ g of either wild-type CdtA-His₆ or CdtC-His₆. The plates were washed, and bound protein was detected as described above for the CELISA. This assay was performed three times.

Saturation kinetics for the subunit-specific antibodies were also determined for the binding of CdtB-His₆ and CdtC-His₆ to CdtA prebound to thyroglobulin.

Concentrations of CdtB-His₆, CdtC-His₆, and the heterodimer ranging from 0 to 4 μ g were added to CdtA-His₆ in triplicate wells on thyroglobulin-coated 96-well plates as described above. The plates were then washed, and bound CdtB-His₆ and CdtC-His₆ were detected using 5×10^{-5} and 1×10^{-6} dilutions of the corresponding IgG preparations and a 1:3,000 dilution of donkey anti-rabbit IgG-horseradish peroxidase conjugate. The plates were washed and developed as described above for the CELISA.

Subunit binding assays. Binding of the mutated CdtA-His₆ to wild-type CdtB-His₆-CdtC-His₆ heterodimer was examined using a competition assay (4). Microtiter plates were prepared as described above for the CdtA-His₆ substitution mutant binding assay, except that the thyroglobulin-coated plates received 10 μ g of wild-type CdtA-His₆/well. Heterotoxins were reconstituted with equimolar concentrations of wild-type CdtB-His₆, wild-type CdtC-His₆, and each of the mutated CdtA-His₆ proteins. Each heterotoxin preparation (3 μ g of total protein) was added to triplicate wells for 1 h at room temperature. The plates were then washed, and bound protein was detected using the anti-CdtB-His₆ and anti-CdtC-His₆ IgG fractions as described above. If a mutation in CdtA-His₆ resulted in a reduction in or loss of binding of the protein to CdtB-His₆ and CdtC-His₆ during the reconstitution step, then the CdtB-CdtC heterodimer was free to bind to wild-type CdtA-His₆ immobilized on the thyroglobulin-coated plate. This resulted in a quantitative increase in the absorbance value. The CdtB-CdtC heterodimer alone binds to the immobilized wild-type CdtA (4). Heterotoxin reconstituted with wild-type CdtA-His₆ was included in every assay to show that it competes against itself. These experiments were repeated three times.

The abilities of the mutated CdtC-His₆ proteins to bind the other individual subunits were determined by examining stoichiometric binding using the thyroglobulin-based ELISA. This modification of the standard assay was developed, characterized, and validated previously (4). Wild-type CdtA-His₆ (4.5 μ g/well) was added to thyroglobulin-coated 96-well microtiter plates as described above. CdtB (4.5 μ g) and either wild-type CdtC or mutated CdtC protein (3.5 μ g) were then added to triplicate wells. After extensive washing bound protein was detected with anti-His₆ Tag monoclonal antibody as described above for the CELISA. An absorbance ratio was calculated by dividing the average (for triplicate wells) absorbance value for the CdtC-His₆ wild-type or mutant protein-containing wells by the average absorbance value for wells containing only bound wild-type CdtA-His₆. An absorbance ratio of 2.0 indicates that only one subunit bound to CdtA on the thyroglobulin. An absorbance ratio of 3.0 indicates that both CdtB and CdtC bound. These experiments were performed a minimum of three times.

The CdtC-His₆ mutated proteins that had absorbance ratios less than 3.0 were further tested for specific binding to wild-type CdtB-His₆. In this assay wild-type CdtA-His₆ (10 μ g/well) was added to thyroglobulin-coated plates and incubated overnight at 4°C. The plates were washed, and 1 μ g of each mutated CdtC-His₆ protein tested was incubated with an equimolar amount of wild-type CdtB-His₆ for 1 h at 4°C. The preincubated preparations were then added to triplicate wells, and the plates were incubated for 1 h at room temperature. Control wells received either no protein, 1 μ g/well of wild-type CdtB-His₆, or 1 μ g/well of wild-type CdtC-His₆. The plates were washed and developed with anti-CdtB IgG, followed by donkey anti-rabbit IgG-horseradish peroxidase conjugate as described above. A duplicate microtiter plate was processed with anti-CdtC IgG.

Heterotoxin assembly. The ability of mutated CdtA-His₆ and CdtC-His₆ proteins to form a stable heterotoxin complex was determined by differential dialysis as described and validated previously (4). In this assay heterotrimers, but not monomers or heterodimers, are retained in dialysis tubing having a molecular mass cutoff of 100 kDa (Spectrum Laboratories, Rancho Dominguez, CA). Each mutant protein was substituted for wild-type CdtA or CdtC in the reconstitution preparation. Following dialysis, 35 μ l of each dialyzed sample was run on a Western blot. Retained proteins were detected with anti-His₆ Tag monoclonal antibody as in the other assays described above. Immunopositive bands on the Western blots were quantified using digitized images with the software program ImageJ, version 1.34 (<http://rsb.info.nih.gov/ij/>). These experiments were performed two times.

To determine if the heterotrimer complexes made with the mutated proteins were properly assembled, their abilities to induce cell cycle arrest were determined by flow cytometry (4). CHO-K1 cells were grown as described above for the CELISA. Cultures were then treated with 10 μ g (total amount) of reconstituted holotoxin/ml of culture medium. Reconstituted holotoxins contained either CdtA-His₆ or CdtC-His₆ mutant proteins and the remaining two wild-type subunits. Cells were exposed to the holotoxin preparations in culture for 36 h. Propidium iodide-stained nuclei were prepared from 1×10^6 cells and were analyzed with a FACSCalibur flow cytometer at the University of Pennsylvania Cancer Center Flow Cytometry and Cell Sorting Shared Resource Facility. The

FIG. 1. Positions of the substitutions for surface-exposed aromatic amino acids in CdtA and CdtC: alignment of the deduced amino acid sequences of *A. actinomycetemcomitans* recombinant CdtA-His₆ and CdtC-His₆. The underlined substitution is not a surface-exposed residue. Identical residues are indicated by shading. Predicted signal cleavage sites and amino termini of the proteins resolved in the crystal structure are indicated by the small and large arrows, respectively. Predicted disulfides are indicated by connecting lines.

response of cells to heterotoxin preparations was considered to be significant if more than 50% of the population was diploid in G₂. The data from 30,000 events were analyzed with ModFit 3.0 (Verity Software House, New Hampshire). The fluorescence-activated cell sorting analyses were repeated two times using independent holotoxin-treated cultures.

Statistical methods. Mean values and standard deviations were plotted where appropriate. The paired *t* test was used where appropriate to predict if experimental values were significantly different from the control value.

Computer modeling. Deduced amino acid sequences of wild-type and mutant *A. actinomycetemcomitans* recombinant CdtA-His₆ and CdtC-His₆ were determined from nucleic acid sequences obtained previously (4). The European Molecular Biology Open Software Suite (EMBOSS release 3.0) (21; <http://emboss.sourceforge.net>) was used to obtain the amino acid sequence alignment of CdtA-His₆ and CdtC-His₆. The crystal structure of *A. actinomycetemcomitans* Y4 Cdt (23) was modeled with the software program UCSF Chimera 1.2197 (20; <http://www.cgl.ucsf.edu/chimera/>). Coordinates were obtained from the Protein Data Bank (accession number 2F2F). Surface-exposed residues and bond distances (in angstroms) were also determined with this program.

RESULTS

Prominent surface-exposed phenylalanine and tyrosine residues in CdtA-His₆ and CdtC-His₆. Similarities in the deduced amino acid sequences of the recombinant *A. actinomycetemcomitans* Y4 CdtA-His₆ and CdtC-His₆ proteins are shown in the alignment in Fig. 1. Forty amino acids, not counting the histidine tails, are identical in the two proteins (CdtA-His₆ and CdtC-His₆ contain 239 and 196 amino acids, respectively). Seven and 10 phenylalanine residues are present in the CdtA-His₆ and CdtC-His₆ subunit proteins, respectively. One phenylalanine residue in each protein is located in the putative signal sequence, and residue F43 in CdtA is not resolved in the *A. actinomycetemcomitans* Y4 Cdt crystal structure (23). Of the remaining phenylalanine residues in CdtA, only F109 and F76 are exposed on the toxin surface. F109 is adjacent to the aromatic patch but is not a prominently exposed residue. F76 is not located near either the aromatic patch or groove regions. Six phenylalanine residues reside on the surface of the CdtC protein (F97, F99, F134, F141, F158, and F170). F97 and F170 are in the groove, and F99 and F141 border the groove.

Nine and seven tyrosine residues are present in CdtA and CdtC, respectively (Fig. 1). One tyrosine residue in CdtC is located in the signal sequence, and residue Y25 in CdtA is not resolved in the Cdt crystal structure. Six tyrosine residues are

exposed on the surface of CdtA (Y101, Y105, Y140, Y181, Y188, and Y189). All of these residues except Y181 border the aromatic patch region as defined in *H. ducreyi* CdtA. Y105 also borders the groove at the junction between CdtA and CdtC. Three tyrosines (Y56, Y61, and Y162) are exposed on the surface of CdtC. Only Y61 is located in the groove.

Alanine was substituted for Y105, Y140, Y188, and Y189 in CdtA-His₆ and for Y56, Y61, F97, F99, F115, F134, F141, and F170 in CdtC-His₆ in independent mutants. A CdtC(F97A, F99A) double mutant was also constructed for comparative studies. These mutants were then used to examine the role of each aromatic residue in (i) subunit and heterotoxin binding to CHO cells and thyroglobulin, a putative Cdt receptorlike glycoprotein, and (ii) subunit binding in heterotoxin assembly. The CdtC(F115A) substitution mutant was made to serve as a protein with a non-surface-exposed residue substitution for comparison in the bioassays.

Effects of aromatic residue substitutions in CdtA and CdtC on direct binding to cells and thyroglobulin. The binding of the isolated mutant proteins to CHO cells was measured using the CELISA and compared to the binding of the wild-type CdtA-His₆ and CdtC-His₆ proteins. We previously showed that wild-type CdtA-His₆ and CdtC-His₆ exhibit saturation binding kinetics with CHO cells (4). Proteins CdtA(Y105A) and CdtA(Y140A) showed moderate but statistically significant decreases in binding to CHO cells (84 and 87% of the wild-type binding, respectively [*P* < 0.01]) (Fig. 2A). Proteins CdtA(Y188A) and CdtA(Y189A) exhibited more dramatic decreases in binding (49 and 48% of the binding of the wild-type proteins). Thus, it appeared that Y188 and Y189 in CdtA are integral to the integrity of the aromatic patch region since loss of either aromatic residue significantly reduced the binding of CdtA to the Cdt receptor on the cell surface. The same binding results were obtained when each of the mutated CdtA-His₆ proteins was first reconstituted with wild-type CdtC-His₆ or with CdtB-His₆ plus CdtC-His₆. An example using CdtA(Y188A) is shown in Fig. 2B. Under the conditions used in this assay there were 26, 24, and 21% reductions in binding of the mutated protein to CHO cells relative to the binding of the wild type when the protein was alone and when it was in a

FIG. 2. Effects of single aromatic amino acid substitutions on the binding of CdtA-His₆ and CdtC-His₆ to CHO cells. (A) Wild-type or mutated CdtA-His₆ proteins (10 μ g/well) were added to wells containing 1.5×10^4 cells. Bound protein was detected with anti-His₆ Tag monoclonal antibody (1:3,000 dilution) and anti-mouse IgG-horseradish peroxidase conjugate (1:3,000 dilution). (B) Wild-type CdtA and CdtA(Y188A) were incubated in reconstitution buffer alone and with CdtC or with CdtB plus CdtC. The preparations (4 μ g of CdtA) were added to wells containing CHO cells as described above for panel A. Bound protein was detected as described above, and absorbance ratios were calculated relative to the binding of CdtA or CdtA(Y188A). (C) Wild-type or mutated CdtC-His₆ proteins run under the same conditions that were used in the experiment whose results are shown in panel A. All samples were run in triplicate. Statistically significant differences between the mutated and wild-type proteins are indicated by asterisks (one asterisk, $P < 0.00001$; two asterisks, $P < 0.001$; three asterisks, $P < 0.01$).

heterodimer and a heterotrimer, respectively. There was a stoichiometric increase in the absorbance values when the CdtA proteins were added to the cells with the other subunits. These data showed that all of the CdtA aromatic amino acid mutants except CdtA(Y105A) were defective in binding to the receptor but not to the other Cdt subunits. Seven of the nine CdtC aromatic residue substitution mutations, including the double mutation, yielded gene products that displayed typical low-level binding to CHO cells compared to the binding of wild-type CdtC-His₆ (Fig. 2C). The CdtC(Y56A) and CdtC(Y61A) mutated proteins exhibited slight increases in binding (121 and 136% of the wild-type binding).

In previous studies we found that CdtA-His₆ binds to thy-

roglobulin with saturation kinetics (4). In contrast, CdtC-His₆ binds very poorly to thyroglobulin in the absence of CdtA. We examined binding of the four CdtA tyrosine substitution proteins to thyroglobulin. These mutated proteins exhibited a pattern of binding nearly identical to that observed with CHO cells (Fig. 3). The levels of binding of CdtA(Y105A), CdtA(Y140A), CdtA(Y188A), and CdtA(Y189A) were 74, 75, 32, and 33%, respectively, of the level of binding of wild-type CdtA.

Effects of aromatic residue substitutions in CdtA and CdtC on binding to wild-type Cdt subunit proteins. In order to assess the abilities of the CdtA-His₆ aromatic amino acid substitution mutant proteins to bind to wild-type CdtB-His₆ and CdtC-His₆,

FIG. 3. Effects of single aromatic amino acid substitutions on the binding of CdtA-His₆ to thyroglobulin. Wild-type or mutated CdtA-His₆ proteins (10 µg/well) were added to wells coated with thyroglobulin. Bound protein was detected as described in the legend to Fig. 2. Statistically significant differences between the mutated and wild-type proteins are indicated by asterisks (one asterisk, $P < 0.00001$; three asterisks, $P < 0.001$).

a competition ELISA was used. Bound CdtB-His₆ and CdtC-His₆ were detected in independent experiments with anti-CdtB and anti-CdtC IgG, respectively (Fig. 4A). The specificity of the antibodies and the specificity of CdtB and CdtC binding to CdtA are shown in Fig. 4B and 4C, respectively. Binding of

wild-type CdtB-His₆ and CdtC-His₆, as well as the heterodimer, to CdtA-His₆ exhibited saturation kinetics. Reconstituted heterotoxin containing wild-type CdtA-His₆ competed against itself. Protein CdtA(Y105A) was the only tyrosine substitution mutant that did not compete with wild-type CdtA-His₆, indicating that it did not bind to CdtB-His₆ and CdtC-His₆. The absorbance values were slightly higher for competing heterotoxin preparations containing CdtA(Y140A), CdtA(Y188A), and CdtA(Y189A) than for heterotoxin made with wild-type CdtA-His₆ (Fig. 4A). These results were most likely due to the lower binding capacity of the mutated proteins than of the wild-type protein. The results were the same when either anti-CdtB or anti-CdtC was used. Of the four CdtA tyrosine residues studied, only the Y105 residue is at the junction between CdtA and CdtC (23).

Heterotoxin reconstituted with the wild-type CdtA-His₆, CdtB-His₆, and CdtC-His₆ subunit proteins is a complex with a 1:1:1 molar ratio. We established that heterotoxin assembly can be quantified as a reproducible stoichiometric increase in absorbance when the three His₆-tagged subunit proteins bind to thyroglobulin (4). The effects of the tyrosine and phenylalanine substitutions in CdtC-His₆ on binding of this subunit to CdtA-His₆ and CdtB-His₆ were evaluated using this assay. The CdtC substitution mutant proteins were preincubated with wild-type CdtB-His₆, and the resulting preparations were added to thyroglobulin precoated with wild-type CdtA-His₆. Three of the nine aromatic amino acid substitution mutant proteins examined, CdtC(Y61A), CdtC(F141A), and the double substitution mutant CdtC(F97A, F99A), had absorbance ratios less than 3.0 (Fig. 5A). Detection of bound protein with

FIG. 4. Effects of single aromatic amino acid substitutions on the binding of CdtA-His₆ to the CdtB and CdtC subunits. (A) Competition assay. Wild-type CdtA-His₆ (4 µg/well) was added to wells precoated with thyroglobulin. Heterotoxin made with wild-type CdtB-His₆, wild-type CdtC-His₆, and either wild-type CdtA-His₆ or a mutated CdtA protein was added to triplicate wells in duplicate plates. The plates were processed as described in the legend to Fig. 2, except that one plate received anti-CdtB IgG and the other received anti-CdtC IgG. Bound IgG was detected with donkey anti-rabbit horseradish peroxidase conjugate. Statistically significant differences between the mutated and wild-type proteins are indicated by asterisks (one asterisk, $P < 0.00001$; two asterisks, $P < 0.0001$; three asterisks, $P < 0.001$; four asterisks, $P < 0.01$). (B) Western blot showing the subunit specificity of the anti-CdtB and anti-CdtC IgG fractions. (C) Saturation curves of the binding of wild-type CdtB-His₆, CdtC-His₆, and the heterodimer to CdtA-His₆-coated thyroglobulin.

FIG. 5. Effects of single aromatic amino acid substitutions on the binding of CdtC-His₆ to wild-type CdtB and CdtC. (A) Wild-type CdtA-His₆ (4 μ g/well) was added to wells precoated with thyroglobulin. Heterodimers made with wild-type CdtB-His₆ and either wild-type CdtC-His₆ or the mutated CdtC proteins were added to triplicate wells. The plates were developed as described in the legend to Fig. 2. Absorbance ratios based on the binding of wild-type CdtA-His₆ to thyroglobulin were calculated as described in Materials and Methods. (B) The three CdtC substitution proteins that exhibited absorbance ratios of less than 3.0 in the experiment whose results are shown in panel A were examined using the thyroglobulin binding assay as described above, except that duplicate plates were developed with anti-CdtB and anti-CdtC IgG as described in the legend to Fig. 4. All samples were run in triplicate. Statistically significant differences between the mutated and wild-type proteins are indicated by asterisks (two asterisks, $P < 0.0001$; three asterisks, $P < 0.001$; four asterisks, $P < 0.01$).

anti-CdtB and anti-CdtC IgG showed that statistically significant smaller amounts of CdtC(Y61A), CdtC(F141A), and CdtC(F97A, F99A) along with wild-type CdtB-His₆ were bound to CdtA-His₆ (Fig. 5B). The reduced amounts of bound CdtB-His₆ observed with these three CdtC mutant proteins suggest that it is the heterodimer rather than the individual subunits that bind to CdtA. The results obtained with the subunit-specific polyclonal antibodies confirmed the differences in binding detected with the anti-His-Tag monoclonal antibody shown in Fig. 5A. However, the CdtB- and CdtC-specific antibodies do not measure the stoichiometric binding of the three Cdt subunits because of their polyclonal nature.

The effects of the amino acid substitutions on subunit assembly were also examined using the dialysis assay. Each of the mutated CdtA-His₆ and CdtC-His₆ proteins was reconstituted with the corresponding wild-type subunit proteins. Of the mutant heterotoxins examined, only those made with CdtA (Y105A), CdtC(Y61A), and CdtC(F141A) failed to form a stable, nondialyzable heterotrimer complex (Fig. 6). The amount of heterotoxin reconstituted with CdtC(F97A, F99A) was substantially reduced (55%) relative to the amount made with wild-type CdtC-His₆. These results support the results obtained with the thyroglobulin ELISA (Fig. 5A).

The mutant heterotoxin preparations tested for heterotrimer formation in the dialysis assay were also examined for the ability to arrest the growth of CHO cells at the G₂/M interphase of the cell cycle to determine if they were biologically active. Heterotoxin preparations reconstituted with CdtA (Y105A), CdtA(Y189A), CdtC(Y61A), CdtC(F141A), and CdtC(F170A) were deficient in blocking cell cycle progression (Fig. 7). Less than 50% of the cells in CHO cell populations exposed to these mutated heterotoxins for 36 h were diploid in G₂ (4n DNA content). Most of these results were expected; the exception was the result for the heterotoxin preparation containing CdtC(F170A) protein. It appears that CdtC(F170A)

may form a heterotrimer that is inactive because of a change in conformation of the assembled complex.

DISCUSSION

Individual surface-exposed phenylalanine and tyrosine residues in the *A. actinomycetemcomitans* Y4 CdtA and CdtC proteins were changed to examine the precise contribution of these amino acids to the binding of the subunits to the receptor and to each other during heterotoxin assembly. Several key observations were made. Most notably, we obtained empirical

FIG. 6. Effects of single aromatic amino acid substitutions on the ability of CdtA-His₆ and CdtC-His₆ to form a Cdt heterotrimer. Each of the mutated CdtA and CdtC proteins was reconstituted with the corresponding wild-type subunit proteins as described in Materials and Methods. The reconstituted preparations were dialyzed for 48 h. Proteins remaining in the dialysis bag were then detected on a Western blot using anti-His-Tag monoclonal antibody. Heterotoxin reconstituted with all three wild-type subunit proteins was examined on a Western blot before (BD) and after (AD) dialysis.

FIG. 7. Abilities of heterotoxins reconstituted with the mutated CdtA and CdtC proteins to arrest the growth of CHO cells. Cell cultures were treated for 36 h with the heterotoxin preparations made as described in the legend to Fig. 6. DNA profiles obtained by flow cytometry of propidium iodide-stained nuclei were analyzed with the ModFit program. Data were expressed as the percentage of the CHO cell population that had a 4n DNA content (diploid in G_2) following treatment with the mutated heterotoxin. The percentages were normalized to the value for CHO cells treated with wild-type toxin. Data for heterotoxin preparations containing mutated CdtA-His₆ and CdtC-His₆ proteins are indicated by open and filled bars, respectively.

evidence that the putative receptor binding domain in CdtA extends from the groove that is formed between CdtA and CdtC across the entire face of the subunit that is oriented 180° from the CdtB subunit (Fig. 8A, yellow residues). The aromatic residues at the periphery of the binding domain (Y105 and Y140) appeared to make a weaker contribution to binding than those closer to the middle of the domain (Y188 and Y189). Nearly identical results were obtained when the mutated proteins were examined to determine their binding to CHO cells, alone and in complexes with CdtB and CdtC, and to thyroglobulin. The data obtained substantially extend the initial findings of Nešić et al. implicating primarily residues W91, W98, W100, and Y102 in *H. ducreyi* CdtA in Cdt binding to the receptor (18). Biochemical analysis was limited to these four heterocyclic and aromatic residues based on interpretations of computer models. Furthermore, only the cumulative effect of the loss of these residues on binding was examined by characterizing a quadruple mutant. The benzenoid rings of Y105, Y188, Y189, and Y140, in addition to those of W90, W97, W99, and Y101, are prominently displayed on the molecular surface of *A. actinomycetemcomitans* Y4 CdtA (Fig. 8B). We showed that loss of single aromatic amino acids in this region can significantly destabilize the binding domain (Fig. 2 and 3).

It is reasonable to expect that CdtA and CdtC would contain similar receptor binding domains if these subunits form two distinct lectin binding regions, as interpreted from computer models. The deduced amino acid compositions and locations of putative disulfides of the CdtA and CdtC subunit proteins of *A. actinomycetemcomitans* Y4 are similar but not identical (Fig. 1) (4). This similarity has been noted for homologs in other members of the Cdt family (10, 13). In spite of the similarities between CdtA and CdtC, a functional aromatic amino acid binding region in CdtC comparable to that in CdtA was not

found. The binding activities of mutated CdtC proteins that had substitutions for the surface-exposed aromatic residues Y56, F97, F99, and F134 were compared. These residues could theoretically form an aromatic amino acid-rich region due to their relatively close proximity in the molecular structure (Fig. 8A). However, no differences between the binding of the mutated proteins to CHO cells and the binding of wild-type CdtC-His₆ were observed. In addition, four additional aromatic amino acid substitutions (Y61A, F115A, F141A, and F170A) and a double substitution (F97A, F99A) failed to affect the binding of the subunit to CHO cells. These data are consistent with our previous observations that CdtC binds to CHO cells in vitro with saturation kinetics, but to a significantly lesser extent than CdtA (4). In the same study we also found that CdtC binds exceptionally poorly to thyroglobulin compared to CdtA. Taken together, these data suggest that *A. actinomycetemcomitans* CdtC may play a relatively minor role in binding of the toxin to its receptor. A heterodimer composed of CdtA and CdtB from *A. actinomycetemcomitans* Y4 is not biologically active (unpublished observations), possibly due to an essential role for CdtC in the uptake of CdtB by the cell (1, 7, 8).

We could not directly study the effects of the four tyrosine substitutions in CdtA on subunit assembly in the thyroglobulin ELISA because this assay relies on the strong binding of CdtA to the glycoprotein. Although, as shown previously (4) and discussed above, CdtB and CdtC bind very poorly to thyroglobulin, both subunits bind with saturation kinetics to CdtA. Therefore, the ability of the CdtA mutants to bind to the other two wild-type subunits was determined using a competition assay. It was shown previously (4) and in this study (Fig. 4C) that a CdtB-CdtC heterodimer specifically binds to CdtA. Of the four CdtA tyrosine mutants examined, only Cdt(Y105A) failed to compete with wild-type CdtA-His₆ for binding to CdtB and CdtC. These results were not surprising since Y105

FIG. 8. Molecular model showing the organization and orientation of the putative Cdt receptor binding domain in CdtA and its relationship to the CdtA-CdtC junction. (A) Surface model of *A. actinomycetemcomitans* Cdt. Residue Y188 protrudes above residue Y105. (B) Ribbon model displaying the locations of the benzenoid rings of the aromatic residues mutated in CdtA and CdtC. Coordinates obtained from Protein Data Bank accession number 2F2F were used in UCSF Chimera to create the models.

is at the junction between CdtA and CdtC. In related subunit assembly experiments with CdtC, two of eight single-aromatic-amino-acid substitution mutants, CdtC(Y61A) and CdtC(F141A), exhibited significantly reduced binding in the thyroglobulin ELISA. Both residues Y61 and F141 are located near the CdtA-CdtC junction, and their loss clearly affected binding of the CdtB-CdtC heterodimer to CdtA. Binding of the CdtC(F97A, F99A) double mutant to CdtA was also reduced even though the two residues are not located at the CdtA-CdtC interface. The most likely explanation for this result is that replacing the two contiguous large phenylalanine residues with alanine significantly alters the folding of CdtC. Replacing either amino acid alone had no effect on subunit binding. The results of the dialysis assay corroborated the results of the subunit binding experiments. Reconstituted preparations made with the appropriate wild-type subunit proteins and either CdtA(Y105A), CdtC(Y61A), or CdtC(F141A) failed to form heterotrimer complexes. These results were strongly supported by the finding that reconstituted preparations made

with these three mutated proteins were extremely deficient in arresting the growth of cells. Based on these results, it appears that residue Y105 in CdtA has a dual role in Cdt binding and subunit assembly. This is most likely because it is located where the aromatic amino acid binding domain extends to the CdtA-CdtC junction. Residues Y61 and F141 in CdtC are essential for proper heterotoxin assembly. The finding that the predominant aromatic residue in CdtC, F170, does not appear to be important for subunit binding even though it is only approximately 7 Å from CdtA was unexpected. However, it is possible that the loss of F170 results in the formation of an unstable or aberrant complex since a heterotrimer made with this mutated protein failed to arrest the growth of cells.

In summary, analysis of the binding properties of the *A. actinomycetemcomitans* CdtA and CdtC proteins having substitutions for single molecular surface-exposed aromatic amino acids (i) increased our understanding of the Cdt receptor binding domain, (ii) provided convincing evidence that the CdtC subunit most likely plays a relatively minor role in cell recognition, and (iii) identified key amino acids important for the binding of CdtA and CdtC during heterotoxin assembly. These findings have important implications for studying the specificity of the receptor for *A. actinomycetemcomitans* Cdt and for developing strategies designed to block the activity of this prominent virulence factor in view of the role of this bacterium in some forms of periodontal disease.

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