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

Linsen Cao,1 Georges Bandelac,2 Alla Volgina,1 Jonathan Korostoff,2 and Joseph M. DiRienzo1*

Departments of Microbiology1 and Periodontics,2 School of Dental Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104-6030

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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 cytoxins (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
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 prominent when these residues are located at the surface of a protein, substitutions were made for surface-exposed aromatic residues in CdtA and CdtC. The effects of these mutations on the binding properties of CdtA and CdtC were assessed by site-directed mutagenesis. Synthetic oligonucleotide primer pairs (Integrated DNA Technologies, Inc., Coralville, IA) were used to change each mutated DNA strand were made using PfuUltra DNA polymerase in PCR (Stratagene, La Jolla, CA). Plasmid pJD9 and pJD2 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- mcrA(mcrBC) Δ(mrr-mrr)] chemically competent cells (Invitrogen, Carlsbad, CA). Nucleotide sequence 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 hsdSB(rK- m-)] 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-His’-Tag monoclonal antibody (Novagen-EMD Biosciences) and anti-mouse immunoglobulin G (IgG)-horseradish peroxidase conjugate (Amersham Pharmacia Biotech, Piscataway, NJ), both at a 1:1000 dilution. Purified wild-type CdtA-His, and CdtD-His, proteins and prestained molecular weight standards (Bio-Rad Laboratories, Hercules, CA) were used as markers. Whole bacterial lysates from E. coli BL21(DE3)(pJD9) and E. coli BL21(DE3)(pJD2) were used as positive controls. Lysate from E. coli BL21(DE3)(pET15b) served as a negative control.

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

<table>
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<tr>
<th>Plasmid</th>
<th>Amino acid change(s)</th>
<th>Primer</th>
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<tr>
<td>pMUT105cdtA</td>
<td>Y105A</td>
<td>CdtA-Y105A-F</td>
<td>5'-GGGCTTTATCCAAATGAGCTTGCAGAGCTTTG-3'</td>
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<td>CdtC-F170A-R</td>
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* Underlined bases are the alanine codons.
Polynthetic 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 1:13,000 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)(pIDAY), E. coli BL21(DE3)(pIDB7), and E. coli BL21(DE3)(pIDC2) 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 Biociences) 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 μg of protein/ml of culture. The final protein preparations were dialyzed to remove urea while promoting protein refolding, passed through 45-μm filters, and quantified with a Micro BCA protein assay kit (Pierce, Rockville, IL) as described previously (4). Purity was assessed by analysis of saturation binding kinetics determined previously (4). The preparations were dialyzed to remove urea while promoting protein refolding, and quantified with a Micro BCA protein assay kit (Pierce, Rockville, IL) as described previously (4).

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 × 10⁴ 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 fixed with a mixture of 2% formaldehyde and 2% glutaraldehyde. After extensive washing with PBS-0.1% Tween 20, bound protein was detected with anti-His protein tested was incubated with an equimolar amount of wild-type CdtB-His6. The mixtures were then incubated with an appropriate dilution 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 μg 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 (5 μg 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 performed three times.

The abilities of the mutated CdtA-His6, proteins to bind to other individual subunits were determined by examining stoichiometric binding using the thyroglobulin ELISA. The reaction kinetics of the subunit was characterized, and validated previously (4). Wild-type CdtA-His6 (4.5 μg/well) was added to thyroglobulin-coated 96-well microtiter plates as described above. CdtB (4.5 μg) and either wild-type CdtC or mutated CdtC protein (3.5 μg) were then added to triplicate wells. After extensive washing bound protein was detected with anti-His-Tag monoclonal antibody as described above for the CELISA. An absorbance ratio was calculated by dividing the average (for triplicate wells containing wells by the average absorbance value for the CdtB-His6, wild-type or mutant protein) containing wells by the average absorbance value for wells containing only 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 of wild-type CdtB-His6. In this assay wild-type CdtA-His6 (10 μg/well) was added to thyroglobulin-coated plates and incubated overnight at 4°C. The plates were washed, and 1 μg 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 or wild-type CdtC-His6, and were not incubated. 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 KD (Spectrum Laboratories, Rancho Dominguez, CA). Each mutant protein was substituted for wild-type CdtA or CdtC in the reconstitution preparation. Following dialysis, 35 μl of each dialyzed sample was run on a Western blot. Retained proteins were detected with anti-His-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 infected with 10 μg (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 × 10⁶ cells and were analyzed with a FACS Calibur flow cytometer at the University of Pennsylvania Cancer Center Flow Cytometry and Cell Sorting Shared Resource Facility. The
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 heterotoxin-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://emboff. sourceforge.net) was used to obtain the amino acid sequence alignment of A. actinomycetemcomitans recombinant CdtA-His6 and CdtC-His6. Amino acid sequences of the recombinant proteins were first reconstituted with wild-type CdtC-His6 and for 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 receptor-like 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 CdtC(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 CdtC(Y189A) exhibited more dramatic decreases in binding (49 and 48% 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
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 thyroglobulin 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,

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**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 μg/well) were added to wells containing 1.5 × 10⁴ cells. Bound protein was detected with anti-His·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 μg 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).
a competition ELISA was used. Bound CdtB-His \(_6\) and CdtC-His\(_6\) 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-His\(_6\) and CdtC-His\(_6\) as well as the heterodimer, to CdtA-His\(_6\), exhibited saturation kinetics. Reconstituted heterotoxin containing wild-type CdtA-His\(_6\) competed against itself. Protein CdtA(Y105A) was the only tyrosine substitution mutant that did not compete with wild-type CdtA-His\(_6\), indicating that it did not bind to CdtB-His\(_6\) and CdtC-His\(_6\). 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-His\(_6\) (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-His\(_6\), CdtB-His\(_6\), and CdtC-His\(_6\) 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 His\(_6\)-tagged subunit proteins bind to thyroglobulin (4). The effects of the tyrosine and phenylalanine substitutions in CdtC-His\(_6\) on binding of this subunit to CdtA-His\(_6\) and CdtB-His\(_6\) were evaluated using this assay. The CdtC substitution mutant proteins were preincubated with wild-type CdtB-His\(_6\), and the resulting preparations were added to thyroglobulin precoated with wild-type CdtA-His\(_6\). Three of the nine aromatic amino acid substitution mutant proteins examined, CdtC(Y61A), CdtC(Y141A), and the double substitution mutant CdtC(F97A, F99A), had absorbance ratios less than 3.0 (Fig. 5A). Detection of bound protein with

![Graph of absorbance vs. form of CdtA protein](image_url)

**FIG. 3.** Effects of single aromatic amino acid substitutions on the binding of CdtA-His\(_6\) to thyroglobulin. Wild-type or mutated CdtA-His\(_6\) proteins (10 \(\mu\)g/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, \(P < 0.001\)).

![Graph of absorbance vs. form of CdtA protein in reconstituted toxin](image_url)

**FIG. 4.** Effects of single aromatic amino acid substitutions on the binding of CdtA-His\(_6\) to the CdtB and CdtC subunits. (A) Competition assay. Wild-type CdtA-His\(_6\), (4 \(\mu\)g/well) was added to wells precoated with thyroglobulin. Heterotoxin made with wild-type CdtB-His\(_6\), wild-type CdtC-His\(_6\), and either wild-type CdtA-His\(_6\), 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-His\(_6\), CdtC-His\(_6\), and the heterodimer to CdtA-His\(_6\)-coated thyroglobulin.
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 made 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
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 resides 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
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 7Å 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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REFERENCES