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Abstract

The Cdt is a family of gram-negative bacterial toxins that typically arrest eukaryotic cells in the G_0/G_1 or G2/M phase of the cell cycle. The toxin is a heterotrimer composed of the *cdtA, cdtB and cdtC* gene products. Although it has been shown that the CdtA protein subunit binds to cells in culture and in an enzyme-linked immunosorbent assay (CELISA) the precise mechanisms by which CdtA interacts with CdtB and CdtC has not yet been clarified. In this study we employed a random mutagenesis strategy to construct a library of point mutations in *cdtA* to assess the contribution of individual amino acids to binding activity and to the ability of the subunit to form biologically active holotoxin. Single unique amino acid substitutions in seven CdtA mutants resulted in reduced binding of the purified recombinant protein to Chinese hamster ovary cells and loss of binding to the fucose-containing glycoprotein, thyroglobulin. These mutations clustered at the 5'- and 3'-ends of the *cdtA* gene resulting in amino acid substitutions that resided outside of the aromatic patch region and a conserved region in CdtA homologues. Three of the amino acid substitutions, at positions S165N (mutA81), T41A (mutA121) and C178W (mutA221) resulted in gene products that formed holotoxin complexes that exhibited a 60% reduction (mutA81) or loss (mutA121, mutA221) of proliferation inhibition. A similar pattern was observed when these mutant holotoxins were tested for their ability to induce cell cycle arrest and to convert supercoiled DNA to relaxed and linear forms *in vitro*. The mutations in mutA81 and mutA221 disrupted holotoxin formation. The positions of the amino acid substitutions were mapped in the *Haemophilus ducreyi* Cdt crystal structure providing some insight into structure and function.

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Characterization of point mutations in the *cdtA* gene of the cytolethal distending toxin of *Actinobacillus actinomycetemcomitans*

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Summary

The Cdt is a family of gram-negative bacterial toxins that typically arrest eukaryotic cells in the G_0/G_1 or G2/M phase of the cell cycle. The toxin is a heterotrimer composed of the *cdtA*, *cdtB and cdtC* gene products. Although it has been shown that the CdtA protein subunit binds to cells in culture and in an enzyme-linked immunosorbent assay (CELISA) the precise mechanisms by which CdtA interacts with CdtB and CdtC has not yet been clarified. In this study we employed a random mutagenesis strategy to construct a library of point mutations in *cdtA* to assess the contribution of individual amino acids to binding activity and to the ability of the subunit to form biologically active holotoxin. Single unique amino acid substitutions in seven CdtA mutants resulted in reduced binding of the purified recombinant protein to Chinese hamster ovary cells and loss of binding to the fucose-containing glycoprotein, thyroglobulin. These mutations clustered at the 5′- and 3′-ends of the *cdtA* gene resulting in amino acid substitutions that resided outside of the aromatic patch region and a conserved region in CdtA homologues. Three of the amino acid substitutions, at positions S165N (mutA81), T41A (mutA121) and C178W (mutA221) resulted in gene products that formed holotoxin complexes that exhibited a 60% reduction (mutA81) or loss (mutA121, mutA221) of proliferation inhibition. A similar pattern was observed when these mutant holotoxins were tested for their ability to induce cell cycle arrest and to convert supercoiled DNA to relaxed and linear forms *in vitro*. The mutations in mutA81 and mutA221 disrupted holotoxin formation. The positions of the amino acid substitutions were mapped in the *Haemophilus ducreyi* Cdt crystal structure providing some insight into structure and function.

Introduction

The cytolethal distending toxin (Cdt) of the periodontal pathogen *Actinobacillus actinomycetemcomitans* is a typical member of a family of secreted gram-negative bacterial protein toxins that classically arrest the growth of many types of eukaryotic cells or cell lines at either the G_0/G_1 or G2/M phase of the cell cycle (reviewed in references Pickett and Whitehouse, 1999; Young and Schauer, 2000; Cortes-Bratti et al., 2001a; De Rycke and Oswald, 2001; Lara-Tejero and Galán, 2002; De Rycke and Ducommun, 2003; Ohara et al., 2004; Heywood et al., 2005; Oswald et al., 2005). Biologically active toxin, from many of the diverse bacterial genera that express the Cdt, is a heterotrimer composed of approximately 18–25 (CdtA), 31 (CdtB) and 21 (CdtC) kDa protein subunits expressed from a polycistronic operon (Akifusa et al., 2001; Lara-Tejero and Galán, 2001; Saiki et al., 2001; Mao and

DiRienzo, 2002; Lee et al., 2003; Shenker et al., 2004). One of the many intriguing characteristics of the *cdt* genes is that they appear to have a eukaryotic rather than prokaryotic heritage. The *cdt* gene products exhibit deduced amino acid sequence and structure/function similarities (albeit weak) to those of eukaryotic proteins. Furthermore, introns have been identified in the *cdt* operon of *A. actinomycetemcomitans* (Tan et al., 2005). The presence of introns, typical of eukaryotic genes, is a rare occurrence in the eubacteria.

The toxic component of the holotoxin appears to be the CdtB protein subunit which has similarity, based on position-specific iterated (PSI) BLAST (Altschul et al., 1997) comparisons, to mammalian type I deoxyribonucleases (Elwell and Dreyfus, 2000; Lara-Tejero and Galán, 2000; Lara-Tejero and Galán, 2002). Purified recombinant CdtB exhibits nicking or relaxation activities, *in vitro*, when incubated with supercoiled DNA (Elwell and Dreyfus, 2000; Elwell et al., 2001). DNA damage in the form of double-stranded breaks, as assessed by pulsed-field gel electrophoresis (PFGE), was detected in sensitive cell types exposed to the holotoxin (Cortes-Bratti et al., 2001b; Frisan et al., 2003; Kanno et al., 2005). Mutating conserved DNase I active site residues in CdtB resulted in the loss of both *in vitro* nuclease activity and *in vivo* cell cycle arrest (Elwell and Dreyfus, 2000; Lara-Tejero and Galán, 2000).

The CdtA protein subunit also appears to be related to eukaryotic proteins. Based on the initial observations reported by Hofmann et al. (2000) and Hassane et al. (2001), amino acid sequence threading analysis (Lara-Tejero and Galán, 2001) and a reverse position-specific (RPS)-BLAST search of the conserved domain (CD) database (Mao and DiRienzo, 2002) functional similarity was noted between a 60–88 sequence of amino acids in CdtA and the B chain of members of the ricin/abrin A–B toxin family. The B chain of ricin acts in a lectin-like interaction attaching to carbohydrate receptors on the cell surface to promote uptake of the A chain of the toxin (Rutenber et al., 1987). Data from immunofluorescence experiments (Mao and DiRienzo, 2002; Akifusa et al., 2005; McSweeney and Dreyfus, 2005; Kanno et al., 2005) and enzyme-linked immunosorbent assays on live cells (CELISA) (Lee et al., 2003) experiments showed that purified recombinant CdtA binds to cells in culture.

A specific eukaryotic connection for the CdtC subunit protein has not been found. However, CdtC has significant amino acid sequence similarity with CdtA (40% for the *Campylobacter jejuni* deduced amino acid sequences; Pickett and Whitehouse, 1999; Lee et al., 2003) suggesting a common function. Purified recombinant CdtC also binds to HeLa (McSweeney and Dreyfus, 2005) and HEp-2 (Akifusa et al., 2005) cells in culture and to HeLa cells in the CELISA (Lee et al., 2003). It has recently been shown that both CdtA and CdtC from *Escherichia coli* are carbohydrate-binding proteins that recognize N-linked fucose moieties on the surface of HeLa cells (McSweeney and Dreyfus, 2005).

The Cdt of Haemophilus ducreyi has been crystalized (NeŠić et al., 2004). The crystal structure supports a ternary complex having three interdependent molecular interfaces. The CdtA and CdtC subunits interact to form two ricin-like lectin domains separated by a deep groove. There is a highly aromatic surface on the CdtA face of the groove. Mutating four aromatic residues (quadruple mutant) in the aromatic patch domain of the CdtA subunit produced a non-toxic reconstituted holotoxin.

Mutagenesis approaches are invaluable for studying the relationship between structure and function. Insertional inactivation of the CdtA gene using antibiotic resistance gene cassette or transposon (Tn*phoA*, Tn5) insertions has been used to demonstrate that this subunit is an important component of the holotoxin (Scott and Kaper, 1994; Lewis et al., 2001; Mao and DiRienzo, 2002; Young et al., 2004). In-frame deletions and site-directed mutagenesis of *cdtA* have provided more detailed information about the role of putative functional regions in

the protein subunit (Lee et al., 2003; Nešić et al., 2004; Saiki et al., 2004). However, there is still a great deal to be learned about the molecular details of the interactions between CdtA and the CdtB and CdtC subunits and between CdtA and cell surface receptors.

To advance studies of the function and interactions of the CdtA subunit and to identify single amino acid residues critical for CdtA activity, we made a library of randomly generated point mutations. Mutants with unique single amino acid substitutions were examined for changes in binding of isolated recombinant CdtA to Chinese hamster ovary (CHO) cells. Holotoxin was reconstituted with each mutated *cdtA* gene product and tested for the ability to inhibit CHO cell proliferation, to promote cell cycle arrest and to exhibit DNase I-like nicking activity *in vitro*. Each mutated *cdtA* gene product was also tested for the ability to bind to fucose-containing glycoproteins, to compete with wild-type CdtA for binding to CdtB and CdtC and to form a holotoxin complex. The greater than 90% similarity between the deduced amino acid sequences of the CdtA proteins of *A. actinomycetemcomitans* and *H. ducreyi* (Cope et al., 1997; Sugai et al., 1998; Mayer et al., 1999) allowed us to map the positions of the amino acid substitutions in the *H. ducreyi* Cdt crystal structure. The implications of specific amino acid substitutions on predicted relationships between structure and function are discussed.

Results

Selection of point mutations in cdtA and isolation of the mutants

A library of randomly generated point mutations was constructed in the plasmid vector pET15b. The His-tag DNA sequence was removed from the plasmid vector and six codons for histidine were placed in the reverse polymerase chain reaction (PCR) primer used to generate the mutant sequences (Table 1). These steps ensured that full length *cdtA* gene sequences could be easily identified in the mutant library by screening for the presence of the His-tag on the gene products. The pJDA9 construct (Table 2), encoding wild-type CdtA, was used as the template for mutagenesis and served as a positive control for all experiments. Mutations were introduced at a very low frequency to enhance chances of recovering mutants having a single nucleotide change. Total cell lysates of transformants carrying potential mutated *cdtA* gene sequences were first screened in a CELISA for reduction in binding to CHO cells relative to an *E. coli* lysate containing wild-type CdtA. This initial screening assay facilitated the analysis of a large number of potential mutants and provided a positive selection method for loss of biological activity. Those transformants that demonstrated binding at levels below that of the wild-type recombinant strain where examined for the correct size DNA insert by either restriction endonuclease mapping or by PCR. Those transformants that had the expected size plasmid DNA insert (668 bp) were then checked for expression of the cloned gene by Western blotting employing a monoclonal antibody that recognized the carboxy-terminal His-tag. Plasmid DNA from those transformants that contained an immuno-positive polypeptide, having an apparent molecular weight of approximately 27 kDa, were then subjected to DNA sequencing to confirm and identify the mutation.

Identification and validation of the point mutations

Of 192 transformants, selected at random following mutagenesis, five failed to grow. Sixty-six transformants exhibited binding in the CELISA comparable to that of the negative control. Twenty-six of these 66 transformants had no insert DNA in the plasmid. Of the 40 remaining transformants 21 had very low level expression of CdtA after IPTG induction resulting in a binding-deficient phenotype. As it would not be feasible to recover significant quantities of gene product from these transformants for testing in the bioassays they were not studied further. The plasmid DNA inserts from the remaining 19 transformants were sequenced. Reliable DNA sequence data could not be retrieved, from two to three sequencing runs, from six of the 19 transformants. The remaining 13 transformants expressed a full length *cdtA* open reading frame

(ORF). Two of these transformants (mutA65 and mutA112) each had two nucleotide changes. Only one of these nucleotide changes in both mutA65 and mutA112 resulted in an amino acid substitution. Eleven transformants had a single nucleotide change. Six of these transformants had a corresponding amino acid change. Two transformants (mutA241 and mutA249) had the same nucleotide change (A655G) and corresponding amino acid substitution (T219A). The mutants are listed in Table 3. The *cdtA* ORFs from seven of the transformants that exhibited binding in the CELISA equivalent to that of the wild type (pJDA9) were also sequenced. No nucleotide changes were found.

The hydropathies of the mutant CdtA-His$_6$ proteins were compared with that of the wild-type protein. Hydropathy was increased in the region surrounding the substituted residues in mutA121^{T41A} and mutA241^{T219A} Hydropathy was decreased in the regions surrounding the substituted residues in mutA65^{C197S}, mutA81^{S165N}, mutA112^{F43S}, mutA221^{C178W} and mutA239^{P56Q} The calculated pI of wild-type CdtA-His$_6$ was 8.2164. The pI of mutA65^{C197S} and mutA221^{C178W} was 8.3192. All the other mutant proteins had a pI identical to that of the wild type.

Effect of cdtA point mutations on subunit binding

Wild-type CdtA-His$_6$ demonstrated saturation binding kinetics with CHO cells in the CELISA (Fig. 1A). No difference was observed between the binding of CdtA labelled at either the amino- or carboxy-terminal end of the protein (data not shown). CdtA-His$_6$ was isolated by affinity chromatography from the seven mutants (Table 3) that had confirmed unique single amino acid substitutions. Binding of the isolated mutant proteins to CHO cells was measured in the CELISA and compared with that of the wild-type CdtA-His$_6$ (Fig. 1B). All seven mutant proteins exhibited a statistically significant reduction in binding ($P < 0.005$ for six mutants, $P < 0.01$ for one mutant). CdtA-His$_6$ was also isolated from one of the mutants (mutA99) that had a single nucleotide change (T264A) but no corresponding amino acid substitution (Table 3). No statistically significant reduction in binding, relative to the wild-type CdtA-His$_6$, was observed with the gene product from this mutant (Fig. 1B). The analysis of mutA99 demonstrated that using crude bacterial extracts in the CELISA can give false positive results and explains why CdtA from five of 11 binding-deficient clones had no amino acid substitutions.

A second binding assay based on immobilized fucose-containing glycoproteins in an ELISA was used to support the CELISA results. Cdt subunit protein binding to thyroglobulin was similar to that observed with CHO cells. Wild-type CdtA-His$_6$ demonstrated saturation kinetics (Fig. 2A). Wild-type CdtB-His$_6$ and CdtC-His$_6$ did not bind and bound poorly respectively. All seven CdtA mutant proteins failed to bind to thyroglobulin ($P < 0.005$; Fig. 2B). Binding of CdtA-His$_6$ from mutA99 again was not statistically different than that of the wild-type protein.

Effect of cdtA point mutations on the activities of reconstituted holotoxin

Reconstituted wild-type holotoxin exhibited a dose dependent effect on the proliferation of CHO cells (Fig. 3A). A TD$_{50}$ was obtained with 1 µg ml^{-1} of reconstituted holotoxin. Reconstituted holotoxins were prepared in a 1:1:1 ratio using the mutant CdtA-His$_6$ tagged protein subunits and wild-type subunits CdtB-His$_6$ and CdtC-His$_6$. Cultures of CHO cells were treated with the various mutant CdtA-containing holotoxins for 36 h and cell survival was compared with that of cultures treated with holotoxin reconstituted with the wild-type CdtA-His$_6$ (Fig. 3B). Holotoxins made with protein from four of the mutants (mutA65, mutA112, mutA239 and mutA241), and wild-type holotoxin (CdtA-H), inhibited proliferation of CHO cells by 95–100%. Holotoxin prepared with CdtA-His$_6$ from mutA81 reduced CHO cell proliferation by 60%. Holotoxins reconstituted with protein from mutA121 and mutA221 had

no detectable effect on proliferation (100% of the input cells survived). CHO cells were also treated with double the concentration (5.0 μg ml^{-1}) of holotoxins reconstituted with protein from mutA81, mutA121 and mutA221 (Fig. 3B, bars labelled 5). The difference in the results were negligible. Holotoxin made with protein from mutA99 (no amino acid substitution) inhibited CHO cell proliferation by 100%. To demonstrate that these results were not an artifact or anomaly associated with CdtA, holotoxins were reconstituted with protein from both mutA81 and mutC41 as well as with wild-type CdtA-His$_6$ and the CdtC-His$_6$ protein from mutC41. The *cdtC* gene in mutC41 contains four nucleotide changes, two of which have corresponding amino acid substitutions (Q49L and S173C) in the gene product. Both of these hybrid holotoxins had no effect on CHO cell proliferation (Fig. 3B).

To further support the observation that several of the CdtA-His$_6$ mutant proteins yielded holotoxins that failed to exhibit biological activity, CHO cell cultures were treated with wild-type and mutant CdtA-containing holotoxin and examined by flow cytometry. CHO cell cultures treated for 36 h with increasing concentrations of wild-type holotoxin showed a dose dependent increase in the percent of cells having a 4n DNA content indicative of arrest at the G$_2$/M phase (Fig. 4A panels a–e). Greater than 90% of the cells (1 × 10^6 cells treated) had a 4n DNA content when exposed to 10 μg ml^{-1} of wild-type holotoxin (Fig. 4B). A TD$_{50}$ was obtained with approximately 4 μg ml^{-1} of reconstituted holotoxin. FACS analysis of CHO cell cultures treated with holotoxin reconstituted with each of the mutant CdtA-His$_6$ protein subunits gave results that paralleled exactly those of the proliferation assay (Fig. 4C). The percentage of CHO cells having a 4n DNA content following exposure to holotoxins made with mutant CdtA-His$_6$ protein from mutA81 (panel b), mutA121 (panel e) and mutA221 (panel f) was 44, 10 and 15, respectively, compared with 66% for cells treated with wild-type toxin (Table 4). Exposure of CHO cells to equivalent concentrations of holotoxins made with mutA65, mutA112, mutA239 and mutA241 CdtA-His$_6$ proteins yielded 70–79% of the cell population with a 4n DNA content. Only 15% of the cell population had a 4n DNA content following treatment with holotoxin made with wild-type CdtA-His$_6$ and CdtB-His$_6$ and *cdtC* gene product from mutC41 (panel i).

It has been shown that wild-type or properly assembled holotoxin has diminished *in vitro* DNA nicking activity presumably due to inhibition of CdtB by the N-terminal tail of correctly assembled CdtC (Nešić et al., 2004). Taking advantage of this semi-quantitative approach, holotoxins reconstituted with recombinant wild-type CdtA-His$_6$ and the CdtA-His$_6$ mutant proteins were examined for the ability to convert supercoiled plasmid DNA to linear and relaxed forms. Holotoxin composed of wild-type subunit proteins had no supercoiled DNA nicking activity relative to CdtB-His$_6$ alone (Fig. 5A). The nicking activity of CdtB-His$_6$ was not affected when this subunit was mixed with either CdtA-His$_6$ or CdtC-His$_6$. CdtA-His$_6$ and CdtC-His$_6$ proteins, alone and in combination, did not display DNA nicking activity. Holotoxins prepared with mutant CdtA-His$_6$ proteins from mutA121 and mutA221 converted all the supercoiled DNA to relaxed and linear forms (Fig. 5B). This was comparable to the nicking activity observed with the free CdtB-His$_6$ subunit and was suggestive of altered holotoxin reconstitution. Holotoxins made with mutant CdtA-His$_6$ proteins from mutA65, mutA81, mutA112, mutA239 and mutA241 showed varying degrees of inhibition of DNA nicking activity. These data indicated that holotoxin reconstitution was altered but not to the extent that CdtB DNA nicking activity was completely inhibited. As expected, the nicking activity of holotoxin made with protein from mutA99 (no amino acid substitution) was inhibited indicative of proper (wild-type) holotoxin assembly. Holotoxin made with mutC41 protein did not exhibit DNA nicking activity (Fig. 5B). However, when both mutA81 and mutC41 were used to make reconstituted holotoxin all of the supercoiled DNA was converted to relaxed form. These results with the CdtA-His$_6$ mutant proteins followed the pattern of inhibition or loss of inhibition of activity observed in the other functional assays.

Two additional methods were used to determine if the mutant CdtA-His$_6$ proteins formed reconstituted holotoxin. In the first method, the thyroglobulin ELISA, shown in Fig. 2 that is based on the binding of CdtA to fucose-containing glycoproteins (McSweeney and Dreyfus, 2005), was used to evaluate the ability of the mutant proteins to compete with wild-type CdtA-His$_6$ for binding to CdtB-His$_6$ and CdtC-His$_6$. Results from this assay using combinations of wild-type subunit proteins showed that the binding of the subunits was specific and additive (Fig. 6A). An increase in the absorbance following binding of CdtB-His$_6$ to CdtA-His$_6$ in the absence of CdtC-His$_6$ was not statistically significant (absorbance ratio of 1.2). However, the increase in absorbance following binding of CdtC-His$_6$ to CdtA-His$_6$ in the absence of CdtB-His$_6$ was significant ($P < 0.01$) and had an absorbance ratio of 1.5. When all three subunit proteins were combined the increase in absorbance was statistically significant ($P < 0.005$) and the absorbance ratio was 3.0. These results demonstrated that all three subunits were bound and that CdtA and CdtC binding was required for binding of CdtB. In competition experiments CdtA-His$_6$ from mutA99, mutA112, mutA121 and mutA239 showed no increase in absorbance (absorbance ratios 1.0–1.4) relative to wild-type CdtA alone suggesting that these mutant proteins formed holotoxin (Fig. 6B). CdtA-His$_6$ from mutA65, mutA81, mutA221 and mutA241 had absorbance ratios below 3.0 (1.8–2.6) suggesting that they may form aberrant or partial holotoxin complexes.

A second assay for reconstitution was based on the size-dependent retention of reconstituted holotoxin (estimated molecular weight of 77 kDa) in dialysis tubing that has a 100 kDa molecular weight cut-off. Holotoxin was reconstituted with wild-type and mutant CdtA-His$_6$ proteins and then dialysed for 48 h at 4°C. The material retained in the dialysis bag was then examined on Western blots. Figure 7A shows that the individual Cdt subunit proteins (estimated molecular weights of 21–31 kDa) and preincubated mixtures of wild-type CdtA-His$_6$ and CdtC-His$_6$ (heterodimer) were not retained following dialysis. In contrast, all three wild-type subunit proteins were detected in the dialysis bag, on Western blots, when reconstituted holotoxin (heterotrimer) was used. All three subunit proteins were retained following dialysis (approximately 100% of each protein retained) when CdtA-His$_6$ from mutA121 was substituted for the wild-type protein (Fig. 7B). This result supported heterotrimer formation by this mutant CdtA protein. However, when CdtA-His$_6$ from mutA221 was used to form holotoxin greater than 75% of CdtB and CdtC and 46% of CdtA were lost from the bag following dialysis (Fig. 7B). Approximately 38%, 31% and 64% of CdtA, CdtB and CdtC, respectively, were not retained when CdtA-His$_6$ from mutA81 was used to make holotoxin. Reconstituted mixtures containing the other CdtA-His$_6$ mutant proteins, which were all active in the bioassays, were trapped in the dialysis bag (data not shown). These results were in good agreement with those from the competition assay. A mixture of CdtA-His$_6$ from mutA121, CdtC-His$_6$ from mutC41 and wild-type CdtB-His$_6$ was not trapped following dialysis indicating that a holotoxin complex was not formed by this combination of mutant subunits.

Mapping amino acid substitutions that alter CDT activity in the holotoxin structure

The amino acid sequences of CdtA from *A. actinomycetemcomitans* and *H. ducreyi* are greater than 91% similar (Cope et al., 1997; Sugai et al., 1998; Mao and DiRienzo, 2002). Figure 8 shows an alignment of the *A. actinomycetemcomitans* and *H. ducreyi* CdtA (GenBank Accession number U53215) deduced amino acid sequences. A single gap was introduced in the *A. actinomycetemcomitans* CdtA sequence at the position of amino acid residue 64 to obtain the alignment. There are 18 conserved amino acid differences between the *A. actinomycetemcomitans* and *H. ducreyi* native CdtA. Three of the *A. actinomycetemcomitans* CdtA mutations (mutA121, mutA112 and mutA239) were at positions that had conserved amino acid differences relative to the *H. ducreyi* CdtA. The other four CdtA mutations were at positions that were identical in the two protein sequences. However, due to the gap in the *A. actinomycetemcomitans* sequence at position 64 the *H. ducreyi* sequence is

out of register by one amino acid after this position. Two of the mutants (mutA65 and mutA221) contained a substitution for a cysteine residue at positions C179 and C198, respectively, in the *A. actinomycetemcomitans* sequence.

Recent publication of the crystal structure of the *H. ducreyi* Cdt (NeŠić et al., 2004) provided a model for mapping the positions of the substituted residues that alter CdtA binding and holotoxin activity. The crystal structure of the *H. ducreyi* Cdt is shown in Fig. 9. Panels A–C illustrate the relationship of the CdtA, CdtC and CdtB subunits respectively. The seven unique mutations in the *A. actinomycetemcomitans* CdtA are mapped on the structure (Fig. 9D). The three mutants mutA121, mutA112 and mutA239, with amino acid substitutions corresponding to the *H. ducreyi* CdtA positions I41, P43 and S56, respectively, contained substituted residues at the amino terminal end of the protein that was not resolved in the crystal structure. All three of these mutants had a binding-deficient phenotype and were the strongest competitors (absorbance ratio ≈ 1) of wild-type CdtA binding to CdtB and CdtC. Only one of these amino terminal point mutations (mutA121^{T41A}), corresponding to position I41 (underlined) in the *H. ducreyi* CdtA protein, also abolished proliferation inhibition activity and the ability to send cells into G2/M arrest when incorporated into reconstituted holotoxin. The other four mutations (mutA65^{C197S}, mutA81^{S165N}, mutA221^{C178W} and mutA241/249^{T219A}) were closer to the carboxy-terminal end of the CdtA protein. All of these mutants had a binding-deficient phenotype and competed (absorbance ratios 1.8–2.6) against wild-type CdtA for binding to CdtB and CdtC. Two of the carboxy-terminal point mutations (mutA81^{S165N} and mutA221^{C178W}), corresponding to positions S166 and C179 (underlined), respectively, in the *H. ducreyi* CdtA protein, resulted in a reduction or loss of proliferation inhibitory activity and the ability to send cells into G2/M arrest. All seven unique mutations reside outside of the aromatic patch region (Fig. 9E).

Discussion

A number of investigations have provided evidence implicating the CdtA and CdtC subunits in the binding of Cdt to cells. Frisk et al. (2001) found all three recombinant Cdt proteins absorbed to the surface of HEp-2 cells. However, a 17 kDa form of CdtA was the predominant bound species. Direct evidence of CdtA binding to cells was obtained from immunofluorescence experiments (Mao and DiRienzo, 2002; Akifusa et al., 2005; McSweeney and Dreyfus, 2005; Kanno et al., 2005) and CELISA (Lee et al., 2003). These studies examined the ability of purified recombinant CdtA subunit protein and reconstituted holotoxin to bind to CHO and HeLa cells respectively. Deng and Hansen (2003) detected CdtC attached to HeLa cells in cultures treated with a CdtA–CdtC complex. However, they could not detect bound CdtC when the same cells were treated with this subunit alone. McSweeney and Dreyfus (2005) showed that CdtA and CdtC bound to agarose bead-immobilized fucose-containing glycoproteins, such as thyroglobulin. We observed that CdtA-His$_6$ bound with saturation kinetics to thyroglobulin-coated ELISA plates (this study). However, CdtC-His$_6$ bound very poorly under the same conditions unless added to the plates together with CdtA-His$_6$.

Although it is now well established that all three Cdt subunits are required for the toxin to manifest its cell inhibitory effects *in vivo* (Pickett et al., 1994; Sugai et al., 1998; Deng et al., 2001; Frisk et al., 2001; Lara-Tejero and Galán, 2001; Saiki et al., 2001; Mao and DiRienzo, 2002), several studies have shown that CdtB and CdtC reconstituted *in vitro* manifests biological activity. HEp-2 cells exposed to a recombinant *A. actinomycetemcomitans* Cdt reconstituted with CdtB and CdtC were arrested at the G$_2$/M phase of the cell cycle (Akifusa et al., 2001). This result was confirmed with purified recombinant *C. jejuni* Cdt subunit proteins and HeLa cells (Lee et al., 2003). However, cell cycle arrest was reduced by 75% when reconstituted holotoxin lacked CdtA. It seems clear that there is an interaction between CdtA and either or both CdtC and CdtB subunits that is required to produce a holotoxin that has

optimal biological activity (Deng and Hansen, 2003; Lee et al., 2003; Nešić et al., 2004; Shenker et al., 2005). CdtA and CdtC may compete for binding to the same fucose-containing cell surface receptor (McSweeney and Dreyfus, 2005) supporting the possibility that these two subunits act in concert *in vivo* to bind toxin to the cell surface. In addition, CdtC appears to be required for internalization of CdtB (Akifusa et al., 2005). However, the precise mechanisms by which CdtA and CdtC each contribute to toxin binding have not yet been clarified.

Deletion mutants and site-directed mutagenesis methods have been employed to provide more detailed information about the binding specificity of CdtA. An in-frame deletion in the *C. jejuni* CdtA, removing amino acids 126–168, did not affect the ability of the reconstituted toxin to arrest HeLa cells, did not reduce binding of the purified subunit protein to these cells in CELISA and competed for binding to these cells with holotoxin containing wild-type CdtA (Lee et al., 2003). This 43 amino acid region is conserved among CdtA proteins in various bacterial species. Apparently, deletion of this conserved region does not affect the biological activity of the toxin. In another study, a series of in-frame deletion mutants lacking amino acids 19–38, 19–47, 19–57, 19–77 and 19–88 was constructed (Saiki et al., 2004). Deletion mutants CdtAΔ19–38 and CdtAΔ19–47 did not affect the ability of the CdtA mutant proteins to make fully active holotoxin. Nešić et al. (2004) used site-directed mutagenesis to make substitutions for four aromatic amino acids (W91G, W98G, W100G and W102A), in a single mutant, in the aromatic patch region of the *H. ducreyi* CdtA. Recombinant Cdt made with this aromatic patch mutant failed to arrest HeLa cells at the G_2/M phase of the cell cycle but did not disrupt stability of the holotoxin as measured by gel filtration chromatography.

In the current study we employed a random mutagenesis strategy to construct a library of point mutations in *cdtA* to assess the contribution of individual amino acids to the binding activity of the subunit and to the ability of the subunit to form active holotoxin. A random mutagenesis strategy rather than a targeted strategy was used initially because, except for the aromatic patch region, no other binding or functional domains have been identified in CdtA. We obtained seven CdtA mutants, with unique point mutations, that produced subunit proteins that had dramatically reduced binding to CHO cells and thyroglobulin. Three of these mutations (mutA121^{T41A}, mutA112^{F43S} and mutA239^{P56Q}) resulted in amino acid substitutions in the amino-terminal region of the protein (corresponding to *H. ducreyi* CdtA positions I41, P43 and S56, respectively, see Fig. 8). This region (residues 18–56) is disordered in the *H. ducreyi* Cdt crystal structure so it is difficult to make predictions about the relationship between the locations of the amino acid substitutions and binding activity (Nešić et al., 2004 and Fig. 9D. It has been proposed that the amino-terminus of CdtA inserts deep in the groove formed between CdtA and CdtC in the crystal structure and may mimic a receptor peptide (Nešić et al., 2004). However, it is possible that the amino terminus of CdtA is important for formation of the groove and therefore may be essential for recognition of the cell surface receptor. If that is the case the amino acid substitutions in mutA121^{T41A}, mutA112^{F43S} and mutA239^{P56Q} may alter the ability of the amino-terminus to fold properly thus changing or masking the CdtA face of the groove. We did not obtain binding-deficient mutants with amino acid substitutions at positions prior to residue 41. This is in good agreement with the previous observation that in-frame deletion of residues 19–47 did not alter toxin activity (Saiki et al., 2004). In that study a dramatic loss of Cdt activity was observed with the next largest deletion in the series (residues 19–57). It is not surprising that deletion of the amino-terminus up to amino acid 47 does not affect Cdt activity because there are indications that CdtA is post-translationally processed by both *A. actinomycetemcomitans* and *E. coli* (Sugai et al., 1998).

The other four binding-deficient mutants (mutA81^{S165N}, mutA221^{C178W}, mutA65^{C197S} and mutA241/249^{T219A}) contained amino acid substitutions toward the carboxy-terminal region of the protein (corresponding to *H. ducreyi* CdtA positions S166, C179, C198 and T220, respectively, see Fig. 8). No mutants containing amino acid substitutions in the aromatic patch

region were obtained (see Fig. 9D and E). The reason for this result is most likely due to our screening method which selected mutants that had a CdtA binding-deficient phenotype. The aromatic patch mutant reported by NeŠić et al. (2004) lost the ability to arrest HeLa cells but not to affect the ability of the mutant protein to form holotoxin. Our data show that single amino acid substitutions outside of the aromatic patch region can affect the binding of free CdtA to cells and to fucose-containing glycoprotein. Several of these mutations affect but do not abolish the ability of CdtA to bind to the other subunits. Two of these mutations (mutA65^{C197S} and mutA221^{C178W}) replaced cysteine residues (corresponding to *H. ducreyi* CdtA positions C179 and C198, respectively, see Figs 8 and 9F).

Three (mutA81^{S165N}, mutA121^{T41A} and mutA221^{C178W}) of the seven CdtA binding-deficient mutants exhibited significantly altered biological activities yet they apparently formed a holotoxin complex with CdtB and CdtC. These mutations contained substituted amino acids at positions I41 (mutA121^{T41A}) in the amino-terminal region and S166 (mutA81^{S165N}) and C179 (mutA221^{C178W}) in the carboxy-terminal region of the *H. ducreyi* CdtA (see Figs 8 and 9D). The mutation in mutA121 is particularly interesting because the CdtA-His$_6$ gene product was a strong competitive inhibitor of wild-type CdtA-His$_6$ binding to CdtB-His$_6$ and CdtC-His$_6$ yet holotoxin made with this mutant protein lacked biological activity. The amino acid switch alanine for threonine in mutA121 was relatively conservative in terms of potential physical changes in the protein. There was a slight increase in the relative hydropathy around residue 41 of the altered gene product. However, the calculated pI of the mutant protein was identical to that of the wild-type protein. It is possible that the threonine in this position is important for the recognition of the N-linked fucose-containing cell surface receptor. In contrast, a switch from phenylalanine to serine at neighbouring position 43 (mutA112) did not affect the biological activity of the subunit. This was a more radical mutation in which a non-reactive amino acid replaced an aromatic residue.

MutA81 is also an interesting mutant because there were partial effects on the cell proliferation inhibition, cell cycle arrest and CdtB DNA nicking activities of holotoxin made with this CdtA-His$_6$ protein. This partial loss of activity correlated with a reduced ability to form heterotrimer. There was a switch from serine to asparagine at position 165 in mutA81. The change from a neutral to acidic amino acid could be significant. However, there was no change in the calculated pI of the mutant protein compared with that of the wild-type protein. The mutation resulted in a slight decrease in the relative hydropathy surrounding this residue. Position 165 in CdtA in the *H. ducreyi* Cdt crystal structure is close to the end of the conserved region in CdtA proteins (Lee et al., 2003). Other than mutA81^{S165N}, we did not find binding-deficient mutations with amino acid substitutions in this conserved region. These findings support the results obtained by Lee et al. (2003) with their 43 amino acid deletion mutant.

An amino acid substitution at position 178 in CdtA (mutA221) also resulted in the loss of holotoxin activity. This residue (C179) in the *H. ducreyi* crystal structure is not near the putative receptor groove between CdtA and CdtC nor does it appear to be in contact with the CdtB subunit. However, it is likely that an intrachain disulphide bridge between C179 and C198 was disrupted (see Fig. 9F). The substitution in mutA221 was a switch from cysteine to tryptophan. Not only was a reactive cysteine residue removed but it was replaced with a large heterocyclic amino acid. The amino acid switch increased the pI of the protein and decreased the hydropathy of the region around residue 178. However, it is interesting to note that a more reserved cysteine substitution in mutA65^{C197S} did not affect the biological activity of CdtA. This mutant protein had a increased calculated pI (same as that of mutA221^{C178W}) and reduced hydropathy relative to the wild-type protein. Based on the difference between the activities of holotoxins reconstituted with protein from mutA221^{C178W} and mutA65^{C197S}, it appears that a disulphide bridge in this region of CdtA may not be required for optimum Cdt activity. The loss of activity in holotoxin made with protein from mutA221^{C178W} is more likely due to the introduction of

a large reactive amino acid that changes the folding of CdtA so that it no longer forms a holotoxin complex. This is supported by the results of the competition experiments in which this mutant protein was a relatively weak competitive inhibitor of wild-type CdtA-His$_6$ binding to the other subunits and the dialysis experiments.

In summary, we have identified point mutations in the *cdtA* gene of the *A. actinomycetemcomitans* Cdt that reduce or abolish binding of the subunit protein to CHO cells and fucose-containing glycoproteins such as thyroglobulin. These mutations resulted in amino acid substitutions that clustered at the amino- and carboxy-terminal regions of the protein. Three of these point mutations resulted in a statistically significant reduction or loss of biological activity of reconstituted holotoxin. Therefore, mutations that affect subunit binding to cells and receptor-like glycoproteins can reside outside of the aromatic patch region. Several of the point mutations resulted in gene products that formed holotoxin complexes that lost the ability to arrest cell growth. In the case of some, but not all of these mutations, the loss of activity could be due to the inability of the mutant protein to properly assemble. The next step is to use a site-directed mutagenesis approach to determine if these mutations reside in more extensive domains in CdtA critical for proper holotoxin assembly and binding to the cell surface.

Experimental procedures

Plasmids and oligonucleotide primers

Based on the sequence of the *cdt* locus of *A. actinomycetemcomitans* Y4 (Mayer et al., 1999; GenBank Accession number AF006830), synthetic oligonucleotide primer pairs (Table 1) were designed and used to amplify the three *cdt* gene sequences. Plasmid pCDT1 (Mayer et al., 1999), containing the *cdtABC* ORFs cloned in pBluescript II SK(+) (Stratagene, La Jolla, CA) was used as template DNA. Oligonucleotide primers were purchased from Integrated DNA Technologies (IDT, Coralville, IA). Codons for six histidine residues (His-tag) were added to the 3′-end of each CdtA, CdtB and CdtC gene sequence using the reverse primers (bases in bold type in Table 1). Products amplified by PCR were cloned into the NcoI and BamHI sites (underlined bases in Table 1) of pET15b (Novagen, EMD Biosciences, San Diego, CA) so that the His-tag in the vector was removed. The amplified DNA was used to transform competent *E. coli* DH5α [*sup*E*44* Δ*lacU169* (φ80*lacZ*ΔM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*] (Invitrogen, Carlsbad, CA). Transformants were selected on Luria–Bertani (LB) agar plates containing 75 μg ml^{-1} of ampicillin. Positive clones were identified by the restriction endonuclease digestion pattern. Plasmid DNA was then isolated from one clone containing each Cdt gene and transformed into *E. coli* BL21(DE3) [F$^-$ *ompT hsdS*$_B$ (r$_B^-$ m$_B^-$) *gal dcm* (DE3)] competent cells (Novagen). The DNA sequence of the insert in the selected plasmids pJDA9, pJDB7 and pJDC2 (Table 2) was obtained to confirm that the *cdt* gene-containing DNA fragments were cloned in the proper orientation and that no mutations were introduced during PCR. 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.

Construction and screening of a cdtA mutant library

The oligonucleotide primer pair cdtA-F/cdtA-R (Table 1) and pJDA9 DNA (Table 2) were used with the GeneMorph Random Mutagenesis Kit (Stratagene), according to the manufacturer's instructions, to generate a set of mutations, at low frequency, in the *cdtA* gene. Mutated *cdtA* DNA fragments were then cloned into the NcoI and BamHI sites of pET15b and transformed into Library Efficiency *E. coli* DH5α Competent Cells (Invitrogen). The transformants were pooled, the plasmid DNA extracted and used to transform *E. coli* BL21 (DE3) competent cells. Individual colonies were transferred to master plates. Each

transformant was grown in 10 ml of LB broth medium containing 75 μg ml^{-1} of ampicillin at 37°C with vigorous shaking. Isopropyl-β-D-thiogalactopyranoside (IPTG) (Sigma Chemicals, St. Louis, MO) was added to a final concentration of 1 mM when the cultures reached late logarithmic phase (OD$_{600}$ = 0.8–1.0). The bacteria were grown an additional 4–5 h, collected by centrifugation (6000 g for 10 min), washed with PBS and lysed at 4°C by sonication (three bursts of 1 min duration at the low power setting; Braun-Sonic 2000, B. Braun Biotech, Allentown, PA). The sonicates were centrifuged at 6000 g for 15 min to collect inclusion bodies. Inclusion body pellets were suspended in 200 μl of PBS containing 6 M urea.

A CELISA, modified from that used by Lee et al. (2003), was used to evaluate binding of the *cdtA* mutants. Briefly, CHO-K1 cells (1.5×10^4 cells well^{-1}1) were used to inoculate 96-well plates (Mao and DiRienzo, 2002). The plates were incubated for 48 h at 37°C in an atmosphere containing 5% CO$_2$. The plates were washed with PBS containing 1 mM each MgCl$_2$ and CaCl$_2$ and the cells fixed with 10% formalin for 15 min at room temperature. The inclusion body fractions from the individual *cdtA* mutant library transformants described above were mixed with 4 volumes of PBS containing 3% BSA (PBS-BSA). These preparations (100 μl) were added to wells in triplicate. The plates were incubated at room temperature for 1 h, washed three times with PBS and then fixed for 15 min at room temperature with 2% formaldehyde and 0.2% glutaraldehyde. The plates were then washed three times with PBS containing 0.1% Tween-20 (PBST) and incubated with PBS-BSA at room temperature for 1 h. A 1:3000 dilution of anti-HisTag monoclonal antibody (Novagen) in PBS-BSA was added to the wells and the plates incubated for 1 h at room temperature. The plates were washed three times with PBS-BSA and incubation was continued with a 1:3000 dilution of anti-mouse IgG horseradish peroxidase conjugate (Amersham Biosciences, Piscataway, NJ) for 1 h at room temperature. The cells were washed three times with PBST followed by the addition of 100 μl of horseradish peroxidase substrate ABTS-100 (Rockland, Gilbertsville, PA). The plates were read in a Perkin Elmer HTS 7000 Bio Assay Reader (Boston, MA) at a wavelength of 405 nm. Absorbance readings were compared with those obtained from triplicate wells containing inclusion body fractions from *E. coli* BL-21(DE3) (pJDA9) and *E. coli* BL-21(DE3) (pET15b). There was no detectable difference in CdtA-His$_6$ binding to formalin fixed and non-fixed CHO cells (data not shown).

Expression of the *cdtA* gene in the individual transformants was assessed by Western blotting. Bacteria collected from 1 ml of IPTG-induced culture were mixed with 100 μl of gel loading buffer (2% SDS, 0.05 M Tris-HCl (pH 6.8), 10% glycerol, 0.01% β-mercaptoethanol), and heated in a boiling water bath for 5 min. Samples (20 μl per lane) were applied to a 10–20% polyacrylamide Tris-HCl Ready Gel (Bio-Rad Laboratories, Hercules, CA). Following electrophoresis, protein bands were transferred to a nitrocellulose membrane. The membrane was blocked with 5% skim milk in TBS buffer [20 mM Tris-HCl (pH 7.6), 0.8% NaCl] for 1 h at room temperature with shaking. The membrane was washed and then incubated with anti-HisTag monoclonal antibody (Novagen), diluted 1:3000 in TBS buffer, for 1 h at room temperature with shaking. The filter was washed and incubated for 1 h with horseradish peroxidase-conjugated anti-mouse IgG (Novagen) diluted 1:3000 in TBS buffer. Immunopositive protein bands were detected with an Enhanced Chemiluminescence Western Blotting Detection Kit (Amersham Pharmacia Biotech, Piscataway, NJ) according to the manufacturer's instructions. Prestained molecular weight standards were obtained from New England Biolabs (Beverly, MA). Lysates from *E. coli* BL-21(DE3) (pJDA9) and *E. coli* BL-21 (DE3) (pET15b) were used as positive and negative controls respectively.

Plasmid DNA was obtained from the mutant library *cdtA* clones using a QIAprep Miniprep kit (QIAGEN, Valencia, CA). The plasmid DNA from each clone was digested with restriction endonucleases NcoI and BamHI according to the manufacturer's instructions. The digested samples were examined on 8% agarose gels to confirm the presence and size of the plasmid

insert. DNA sequence analysis was performed by the Genetics Core Facility at the University of Pennsylvania as described above.

Isolation of recombinant wild-type His$_6$-tagged CdtA, CdtB and CdtC and mutant CdtA subunit proteins

Recombinant clones *E. coli* BL-21(DE3) (pJDA9), *E. coli* BL-21(DE3) (pJDB7), *E. coli* BL-21 (DE3) (pJDC2) and individual clones from the *cdtA* mutant library were used to prepare the three wild-type His-tagged Cdt protein subunits and mutated His-tagged CdtA proteins as described previously (Mao and DiRienzo, 2002). Yield was approximately 2–4 mg protein per 100 ml of culture. The final dialysed protein preparations were passed through 45 micron filters and quantified with the Micro BCA protein assay kit (Pierce, Rockville, IL). Purity was assessed by analysis on 10–20% polyacrylamide gels. 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 MgCl$_2$ and 5 mM imidazole.

Binding of recombinant wild-type His$_6$-tagged CdtA, CdtB and CdtC and mutant CdtA subunit proteins to CHO cells

Saturation binding kinetics were determined for wild-type CdtA-His$_6$, CdtB-His$_6$ and CdtC-His$_6$ using 0–1.25 mg of each protein per millilitre in triplicate in the CELISA. To assess binding activity purified His$_6$-tagged wild-type and mutant proteins (1 μg well^{-1}) were added to triplicate wells in the CELISA. The 96-well plates were prepared and processed as described above for screening of the transformants. These experiments were run a minimum of three times.

Reconstitution and activity of wild-type and mutant CdtA-containing holotoxin

Wild-type holotoxin and holotoxin containing mutant CdtA-His$_6$ proteins were reconstituted as described previously (Mao and DiRienzo, 2002). The recombinant His$_6$-tagged proteins were mixed in equivalent proportions of wild-type CdtA-His$_6$ or mutated CdtA-His$_6$ with wild-type CdtB-His$_6$ and CdtC-His$_6$ and incubated for 1 h at 4°C in reconstitution buffer (10 mM Tris-HCl (pH 7), 100 mM NaCl, 5 mM MgCl$_2$). In some experiments holotoxin was reconstituted with a CdtC-His$_6$ mutant protein. This mutant, mutC41, was obtained by the same methods used for creation of the CdtA mutant library (data not shown). This mutant had the fewest nucleotide changes identified in the CdtC library [four nucleotide changes and two amino acid substitutions (Q49L and S173C)].

Proliferation inhibition

To quantify the effects of reconstituted holotoxin on cell proliferation, CHO cells were seeded in six-well tissue culture plates (300 cells in 3 ml of medium per well in triplicate) as described previously (Mao and DiRienzo, 2002). Cell cultures were incubated for 4 h prior to addition of the toxin to allow the cells to attach to the plates. Holotoxin preparations made with wild-type and mutant CdtA-His$_6$ proteins (2.5 or 5 μg of total protein ml^{-1}) were added to the CHO cell cultures and the plates were incubated for 5–6 days to allow colonies to form. The medium was then removed and the cells were fixed with 10% formalin. After incubation for 5 min, the colonies were stained with crystal violet for 10 min, dried and counted. The number of colonies per well was expressed as colonyforming units (cfu). A dose–response curve for wild-type reconstituted holotoxin was also prepared using 0–2.5 μg of total protein ml^{-1}. Due to limiting amounts of purified proteins samples were run in duplicate wells. These experiments were run twice.

Cell cycle analysis

Cell cycle arrest was determined by flow cytometry as described previously (Kanno et al., 2005). A dose–response curve was obtained with reconstituted wild-type holotoxin using 2.5, 5, 10 and 25 μg ml^{-1} of reconstituted wild-type holotoxin. CHO cells were treated with 10 μg ml^{-1} total protein of holotoxin made with mutant CdtA-His$_6$ proteins. Cells were exposed to the toxin in culture for 36 h. Propidium iodide stained nuclei were analysed on an FACSCalibur flow cytometer at the University of Pennsylvania Cancer Center Flow Cytometry and Cell Sorting Shared Resource facility. The data from 30 000 events were analysed with ModFit 3.0 (Verity Software House, NH).

In vitro *DNase activity*

Supercoiled DNA nicking activity was determined, with minor modifications, as described previously (Elwell and Dreyfus, 2000; Mao and DiRienzo, 2002). Briefly, 1 μg of supercoiled pBluescript II SK(+) DNA was incubated with reconstituted holotoxin (500 ng of each subunit protein per reaction) in 25 mM HEPES (pH 7.0) containing 50 mM MgCl$_2$ at 37°C for 1 h. Holotoxin was reconstituted with wild-type CdtB-His$_6$ and CdtC-His$_6$ and either wild-type CdtA-His$_6$ or one of the CdtA-His$_6$ mutant proteins as described above except that incubation was at room temperature for 1 h. In some reactions CdtC-His$_6$ from mutC41 was used in place of the wild-type subunit.

Glycoprotein ELISA

A glycoprotein ELISA assay was designed based on the immobilized glycoprotein binding assay of McSweeney and Dreyfus (2005). Microtitre plates (96-well) were coated overnight with 75 μg well^{-1} of thyroglobulin (Sigma). The plates were washed three times with PBST followed by the addition of wild-type or mutant Cdt subunit proteins. All proteins were added to triplicate wells at a concentration predetermined for each experiment (described below). Plates were washed, treated with anti-His Tag monoclonal antibody and horseradish peroxidase conjugate and were processed as described above for the CELISA. Saturation binding kinetics were determined for wild-type CdtA-His$_6$, CdtB-His$_6$ and CdtC-His$_6$ using 0–20 μg of each protein per well. In other experiments, various combinations of subunit proteins using 4 μg of CdtA-His$_6$, 4.5 μg of CdtB-His$_6$ and 3.5 μg of CdtC-His$_6$ were added per well to assess holotoxin assembly. In a third set of experiments, 10 μg of each subunit protein and each CdtA-His$_6$ mutant protein was added per well to examine the ability of the mutant proteins to bind to the glycoprotein. Competition experiments were performed by preincubating 4.5 μg of CdtB-His$_6$ and 3.5 μg of CdtC-His$_6$ with 4 μg of each mutant CdtA-His$_6$ protein. Thyroglobulin-coated plates were incubated with 4 μg of wild-type CdtA-His$_6$ protein well^{-1} and the plates were washed three times with PBST. The subunit protein mixtures were then added to the plates and the plates processed as described above. An absorbance ratio was calculated by dividing the average (triplicate wells) absorbance value of the CdtB-His$_6$/CdtC-His$_6$ protein or mutant CdtA-His$_6$ protein wells by the average absorbance value of wells containing only bound wild-type CdtA-His$_6$. The binding experiments were repeated two to three times and, except for the competition assay, were repeated with fetuin-coated plates.

Reconstitution (dialysis) assay

Dialysis membrane with a molecular weight exclusion limit of 100 kDa (Spectrum Laboratories, Rancho Dominguez, CA) was soaked and rinsed in distilled water. Each of the subunit proteins alone (100 μg of protein or mutant protein) or reconstituted heterotrimer (300 μg of total protein) were suspended in a total volume of 1 ml of refolding buffer containing 0.3 M urea. Each sample was dialysed against 800 ml of the same buffer for 48 h at 4°C with two buffer changes. An aliquot (35 μl) of each protein or reconstituted protein sample was then

examined, before and after dialysis, on Western blots as described above. Bands on Western blots were quantified with ImageJ version 1.34 (http://rsb.info.nih.gov/ij/).

Computer analysis

The European Molecular Biology Open Software Suite (EMBOSS 2.9.0) (http://emboss.sourceforge.net) was used to obtain amino acid sequence alignments and to calculate isoelectric points (pI) and hydropathy (Kyte and Doolittle, 1982; Rice et al., 2000). Deduced amino acid sequences of wild-type and mutant *A. actinomycetemcomitans* recombinant CdtA-His$_6$ and CdtC-His$_6$ were from nucleic acid sequences obtained during this study. The *H. ducreyi* CdtA sequence was from GenBank (Accession number U53215).

The crystal structure of the *H. ducreyi* Cdt (NeŠić et al., 2004) was modelled in the software program iMol 0.30 (http://www.pirx.com/iMol/). Coordinates were obtained from the Protein Data Bank (Accession number 1SR4).

Statistical methods

Mean values and standard deviations were plotted where appropriate. The paired *t*-test was used to evaluate the data in some experiments.

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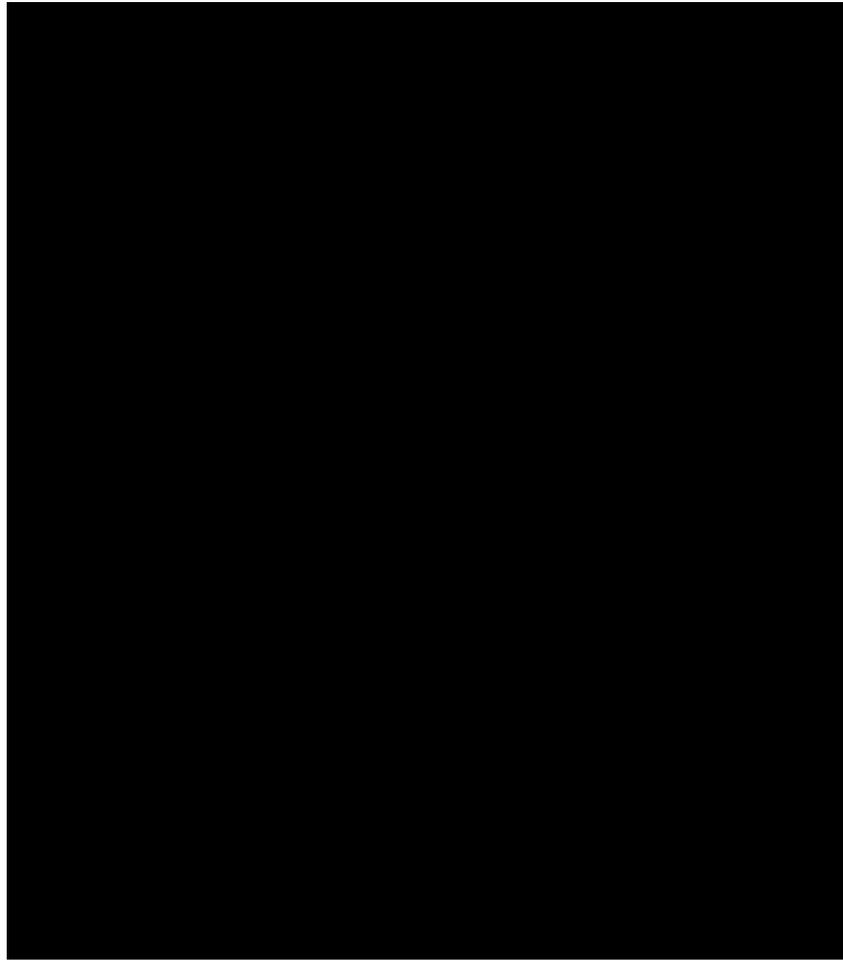
Fig. 1.
Effect of point mutations on CdtA binding to CHO cells. Affinity-purified CdtA mutant proteins were incubated with CHO cells in a CELISA. A. Saturation curves of wild-type recombinant CdtA-His$_6$, CdtB-His$_6$, CdtC-His$_6$ and total soluble protein from *E. coli* BL-21 (DE3) (pET15b) in a CELISA. Bound Cdt protein subunit was detected with anti-HisTag monoclonal antibody (1:3000 dilution) and anti-mouse IgG horseradish peroxidase conjugate (1:3000 dilution). B. Recombinant CdtA-His$_6$ mutant proteins (1 μg well^{-1}) were incubated with CHO cells in a CELISA. Bound protein was detected as in the experiment in A and compared with wells containing wild-type CdtA-His$_6$, CdtB-His$_6$ and CdtC-His$_6$. Amino acid substitutions are shown for each mutant. All samples were run in triplicate. Statistically significant differences between the mutants and wild-type CdtA-His$_6$ are marked with asterisks ($P < 0.005$*, $P < 0.01$**). Results are representative of three trials.
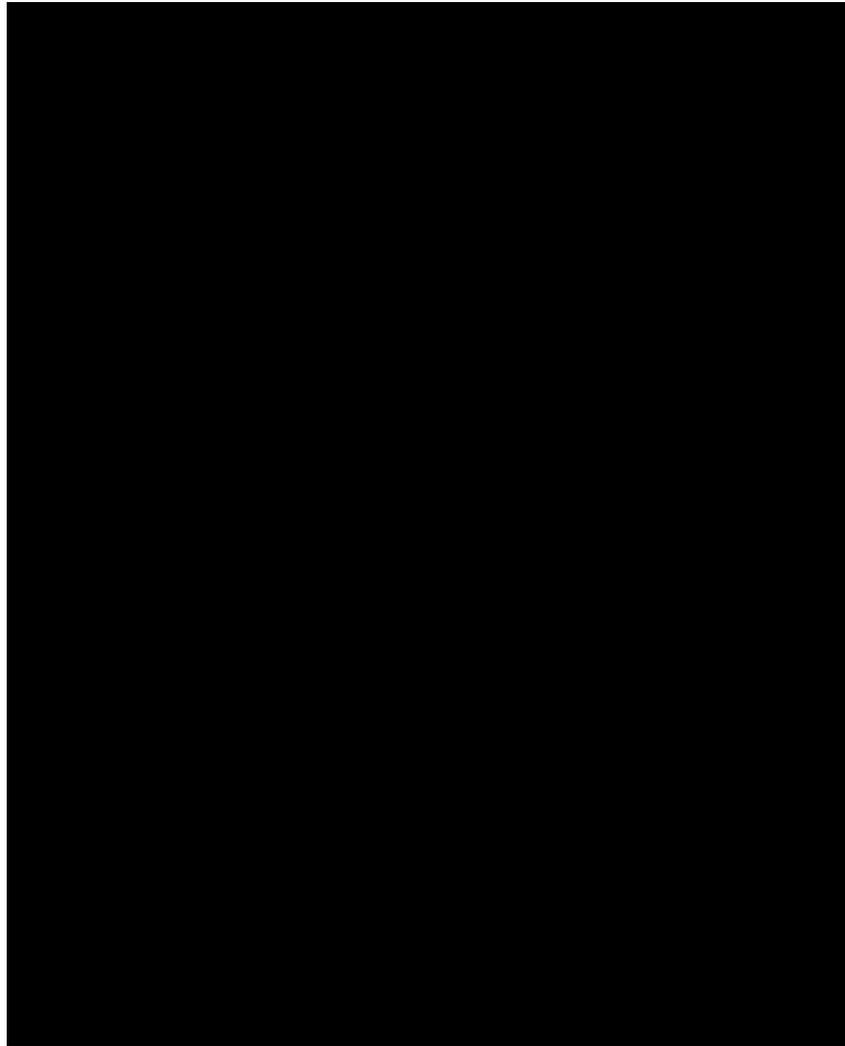
Fig. 2.
Effect of point mutations on CdtA binding to thyroglobulin. A. Saturation curves of the binding of wild-type recombinant CdtA-His$_6$, CdtB-His$_6$, CdtC-His$_6$ to thyroglobulin-coated wells in ELISA. Bound protein was detected as described in the legend to Fig. 1. B. Affinity-purified recombinant CdtA-His$_6$ mutant proteins (10 μg well^{-1}) were added to wells coated with thyroglobulin. Bound protein was detected as in the experiment in A and compared with wells containing 10 μg well^{-1} of wild-type CdtA-His$_6$, CdtB-His$_6$ and CdtC-His$_6$. Amino acid substitutions are designated for each mutant. All samples were run in triplicate. Statistically significant differences between the absorbance values for mutant and wild-type CdtA-His$_6$ are marked with an asterisk ($P < 0.005$). Results are typical of three trials.
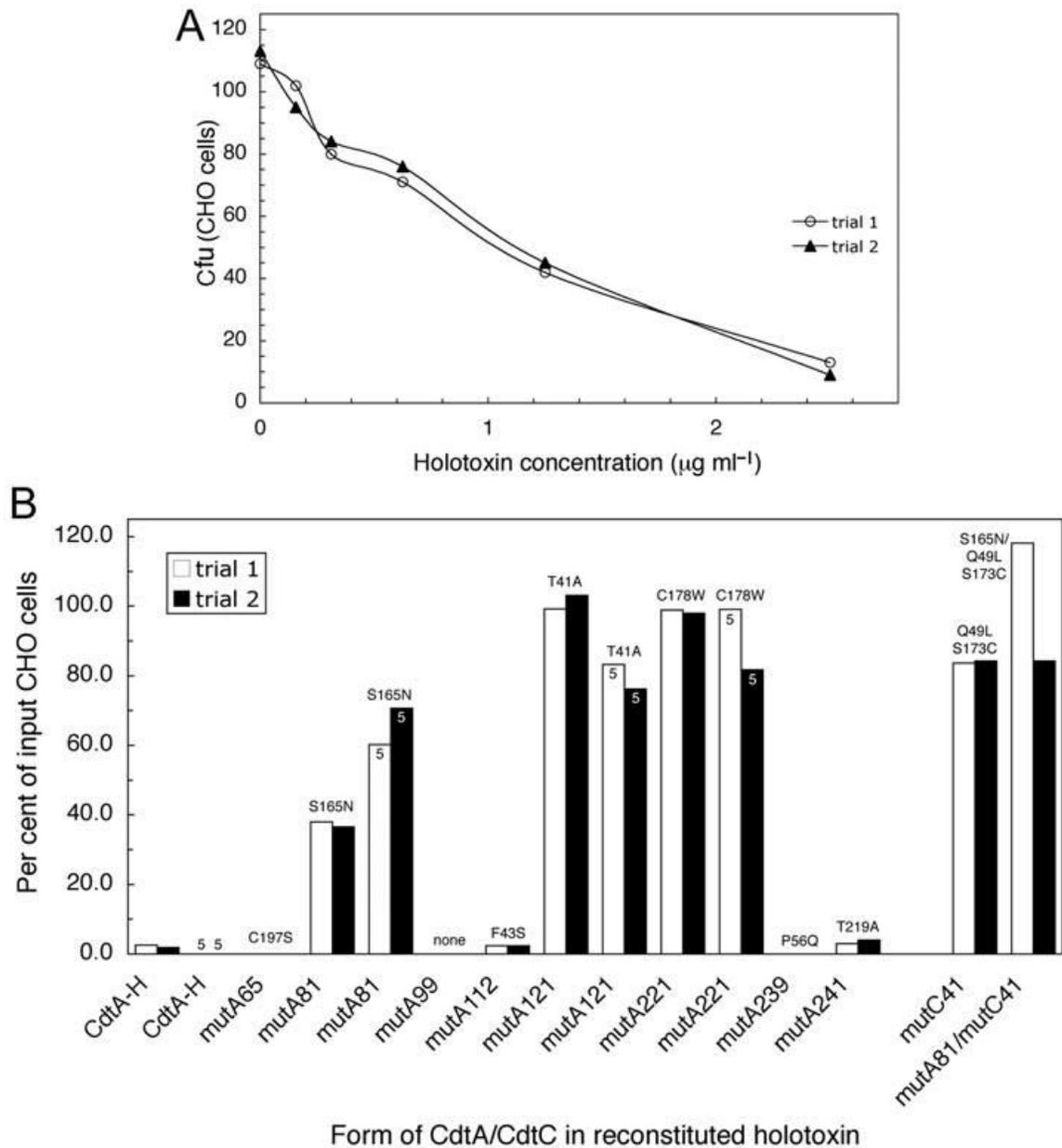
Fig. 3.
Effect of point mutations in CdtA on the proliferation of CHO cells treated with reconstituted holotoxin. A. Wild-type recombinant CdtA-His$_6$, CdtB-His$_6$ and CdtC-His$_6$ (1:1:1 by weight) were preincubated in folding buffer and increasing concentrations of the mixture was added to CHO cell cultures. Cell colonies were stained and counted after 6 days and the data expressed as cfu. B. Holotoxin was reconstituted as in the experiment in A except that recombinant CdtA-His$_6$ mutant proteins were substituted for wild-type CdtA-His$_6$. In some samples a CdtC-His$_6$ mutant (mutC41) was substituted for wild-type CdtC-His$_6$. Amino acid substitutions are shown for each mutant. MutC41 contained two amino acid substitutions. CdtA-H designates holotoxin reconstituted with wild-type CdtA-His$_6$. All samples were run twice in single wells. Twice the amount of CdtA-His$_6$ protein (5 μg ml^{-1}) was used to treat some cultures. Cfu were obtained as in A and the data is expressed as the percent of input CHO cells.
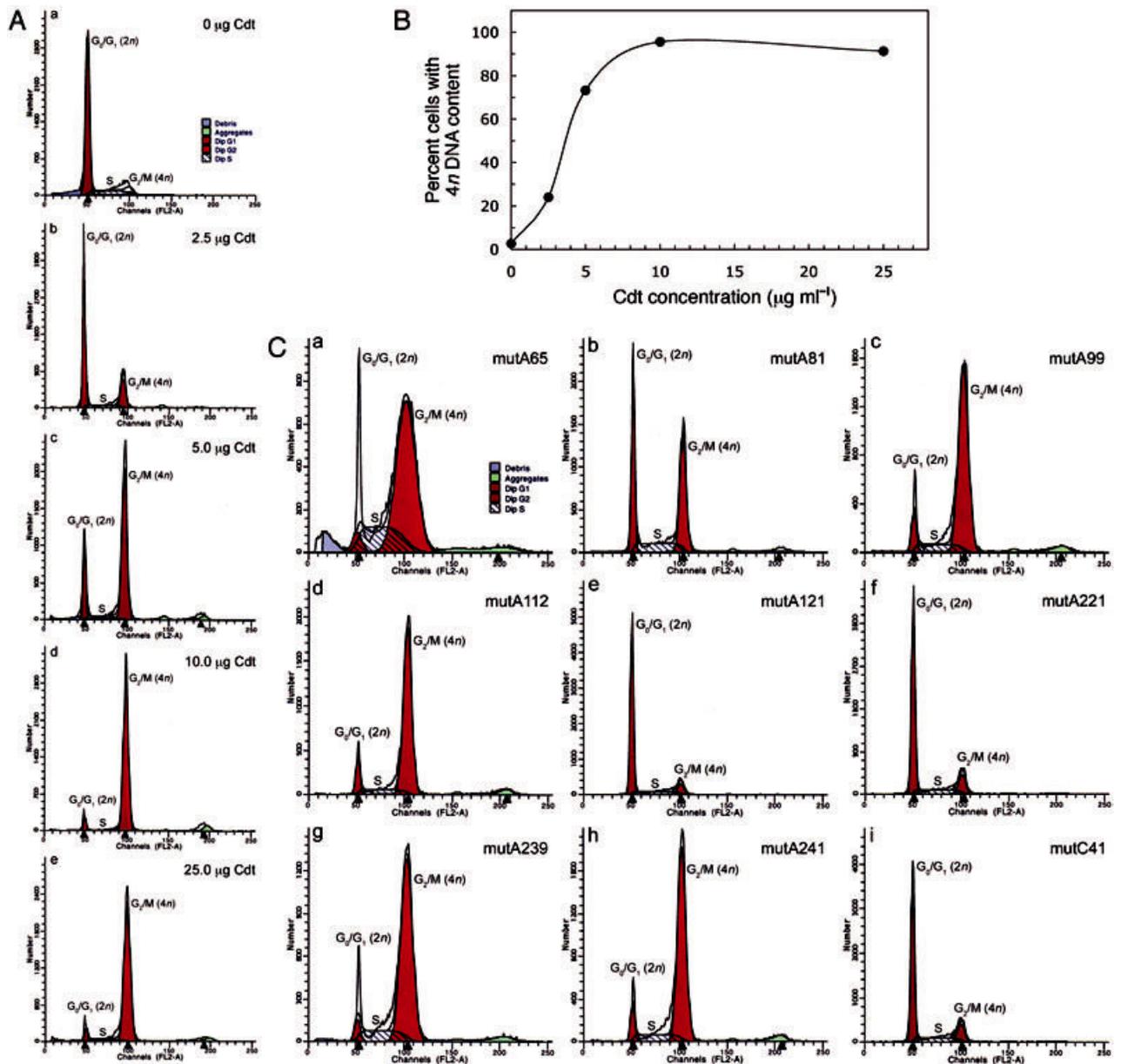
Fig. 4.
Effect of point mutations in the *cdtA* gene on the cell cycle of CHO cells treated with
reconstituted holotoxin. A. CHO cell cultures (1×10^6) were treated with 0, 2.5, 5.0, 10.0 and
25.0 μg of total holotoxin protein ml^{-1}. Holotoxin was reconstituted from wild-type CdtA-
His$_6$, CdtB-His$_6$ and CdtC-His$_6$ (1:1:1) for 36 h post intoxication. DNA profiles obtained by
FACS analysis of propidium iodide stained nuclei are shown (solid line). G0/G$_1$ (2n) and G$_2$/
M (4n) (solid filled) and S (diagonal line filled) peaks where determined by computer analysis.
B. Dose–response curve of the percentage of cells having a 4n DNA content after treatment
with each holotoxin concentration. C. CHO cell cultures were treated as in A except that 10
μg of total mutant CdtA or CdtC holotoxin protein ml^{-1} was used. Panels a, mutA65; b,
mutA81; c, mutA99; d, mutA112; e, mutA121; f, mutA221; g, mutA239; h, mutA241; i,
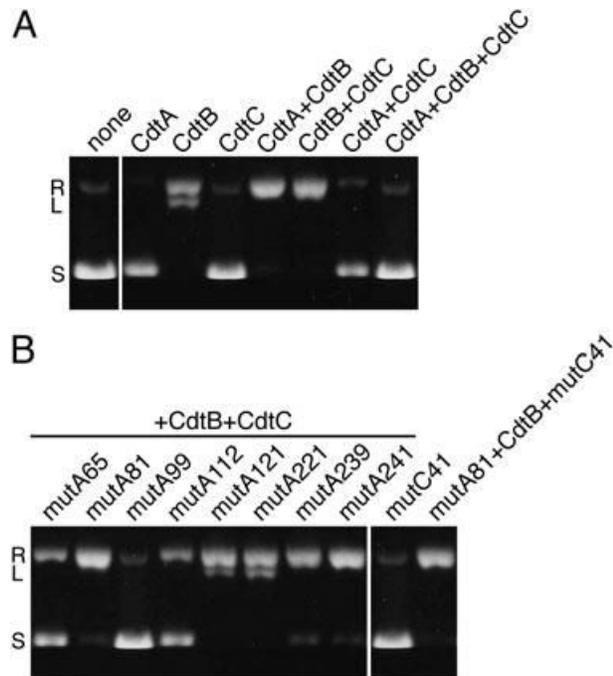mutC41. Results are representative of two experiments.

Fig. 5.
Effect of point mutations in the *cdtA* gene on the *in vitro* DNA nicking activity of reconstituted holotoxin. A. Wild-type Cdt subunit proteins alone and in various combinations were incubated with pBluescript II SK(+) DNA under conditions optimized for CdtB-His$_6$ nuclease activity (*Experimental procedures*). B. Holotoxin prepared with protein from each of the CdtA-His$_6$ mutants and from mutC41 was incubated with plasmid DNA as in A. Samples were examined on 0.8% agarose gels stained with ethidium bromide. Supercoiled (S), linear (L) and relaxed (R) forms of DNA are labelled. Results are representative of three experiments.
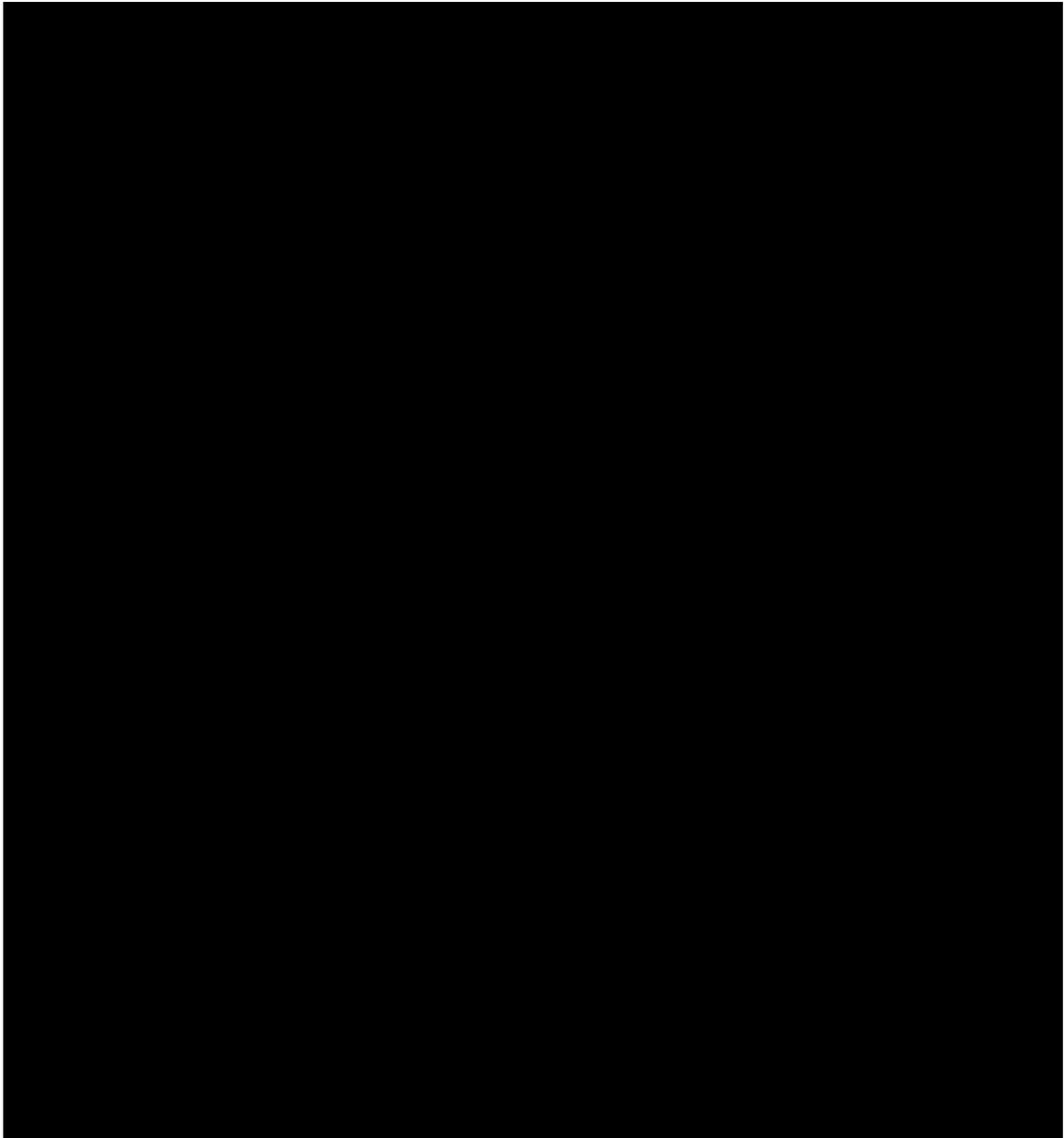
Fig. 6.
Competitive binding of mutant CdtA-His$_6$ proteins to CdtB-His$_6$ and CdtC-His$_6$. A. Subunit, heterodimer and heterotrimer binding to thyroglobulin. ELISA plates were coated with thyroglobulin followed by the addition of individual or a combination of wild-type subunit proteins. B. Competition assay. ELISA plates were coated with thyroglobulin followed by 4 μg well^{-1} of wild-type CdtA-His$_6$. Individual wild-type CdtB-His$_6$ and CdtC-His$_6$ subunit proteins, a preincubated mixture of CdtB-His$_6$ with CdtC-His$_6$ and preincubated mixtures of the mutant CdtA-His$_6$ proteins with wild-type CdtB-His$_6$ and CdtC-His$_6$ were added to the plates. The plates were developed and the absorbance determined as described in the legend to Fig. 1. All samples were run in triplicate. Absorbance ratios based on the binding of wild-type CdtA-His$_6$ to thyroglobulin were calculated as described in *Experimental procedures*. A statistically significant increase in the absorbance values for the homodimers and heterotrimer

relative to that of wild-type CdtA-His$_6$ are marked with an asterisks ($P < 0.005*$, $P < 0.01**$). Results are representative of two experiments.
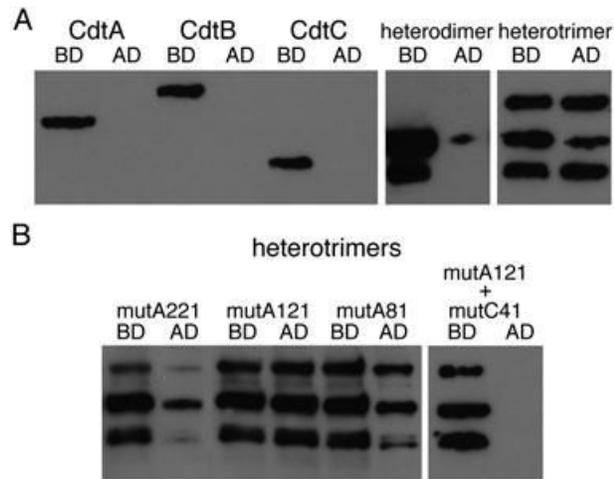
Fig. 7.
Effect of point mutations in the *cdtA* gene on the reconstitution of holotoxin. Individual wild-type subunit proteins and heterotrimers composed of preincubated mixtures of either wild-type or mutant CdtA-His$_6$ proteins were dialysed for 48 h. The protein composition of the material in the dialysis bag, before and after dialysis, was then examined by Western blotting as described in the *Experimental procedures*. BD, before dialysis; AD, after dialysis.
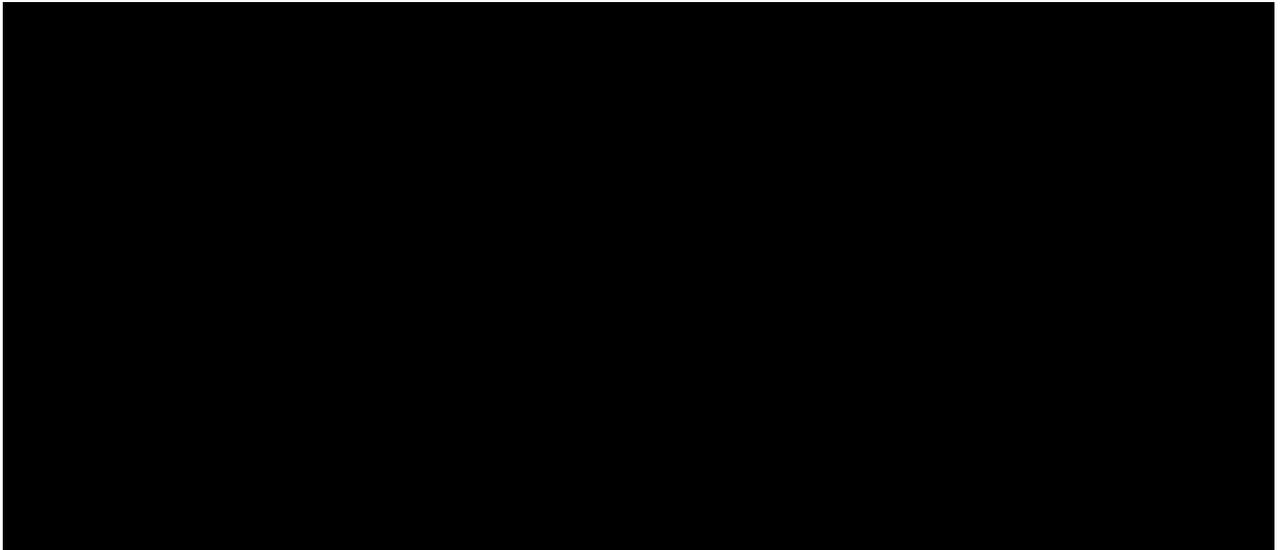
Fig. 8.
Alignment of *A. actinomycetemcomitans* recombinant CdtA-His$_6$ (pJDA9), *H. ducreyi* CdtA (GenBank Accession number U53215) and CdtA-His$_6$ mutant deduced amino acid sequences (Table 3). Identical residues are boxed. The positions of the four amino acid substitutions in the aromatic patch (ap) mutant described in reference 24 are marked. Amino acid resides 18–56 were disordered in the crystal structure (NeŠić et al., 2004). The arrow marks the amino-terminal end of the 17–18 kDa form of CdtA identified in several studies (Frisk et al., 2001;Saiki et al., 2004;Shenker et al., 2004). The solid line between the two sequences marks the region conserved among the various CdtA proteins (Lee et al., 2003).
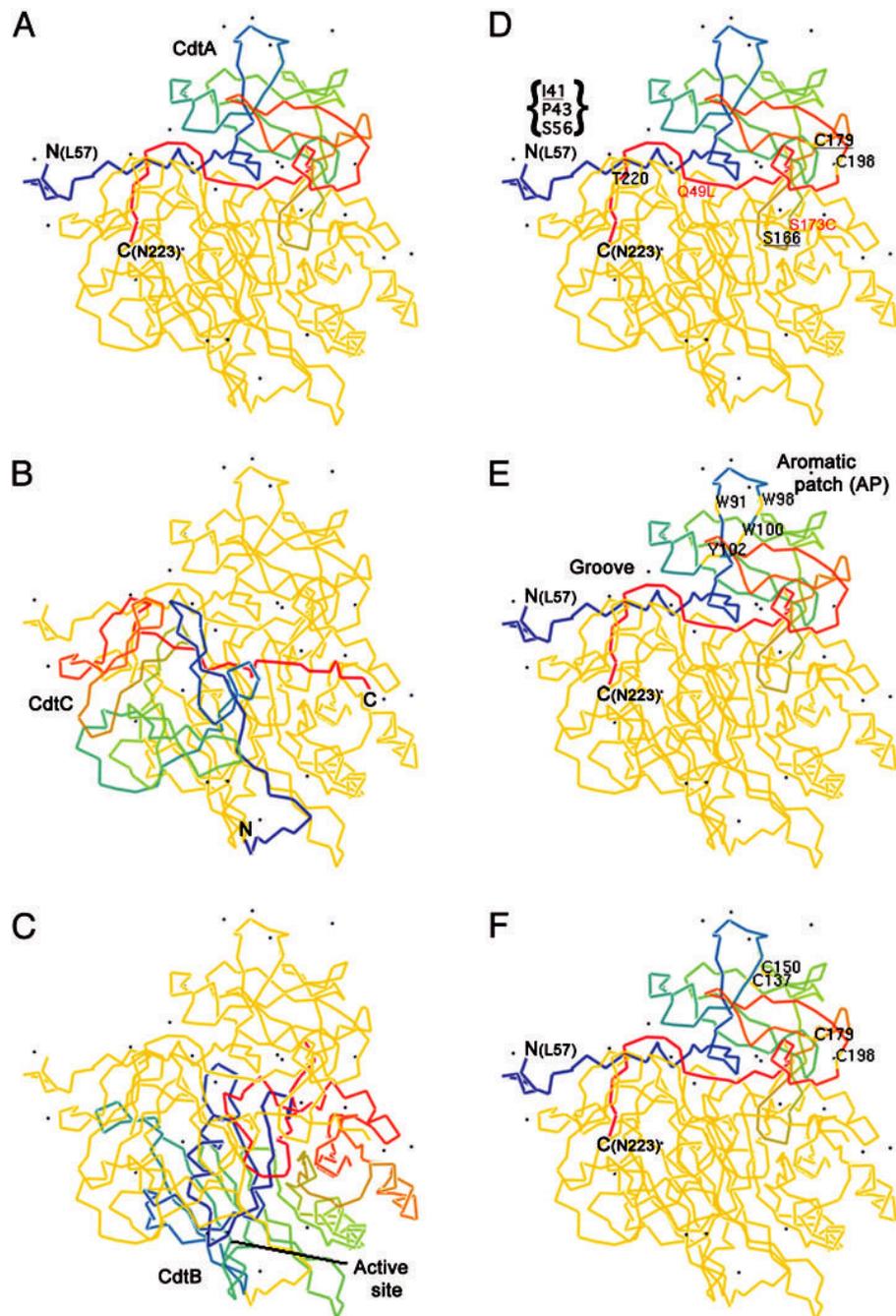
Fig. 9.
Location of the amino acid substitutions of the CdtA-His$_6$ mutant proteins in the crystal structure of the *H. ducreyi* Cdt. The coordinates (Protein Data Bank Accession number 1SR4) for the crystal structure (NeŠić et al., 2004) were computer modelled. A–C. Orientation of the CdtA, CdtC and CdtB subunits respectively. The amino-(N) and carboxy-(C) terminal residues in CdtA and CdtC and the theoretical active site in CdtB are labelled. D. Locations of the seven unique amino acid substitutions (black text) in the *A. actinomycetemcomitans* recombinant CdtA-His$_6$ mutants that affected binding are shown at the positions of the corresponding residues in the *H. ducreyi* sequence. The positions of the three substitutions that resulted in a non-cytotoxic phenotype are underlined. The CdtA used to crystalize the *H. ducreyi* Cdt

contained residues 18–223 to eliminate a putative secretory signal sequence (NeŠić et al., 2004). Residues 18–56 were disordered in the crystal structure. The two amino acid substitutions in the CdtC-His$_6$ mutant (mutC41) are shown in red. E. Location of the four amino acid substitutions in the aromatic patch (AP) region mutant of NeŠić et al. (2004). F. Locations of the four cysteine residues in CdtA. These cysteines are conserved in the *A. actinomycetemcomitans* and *H. ducreyi* CdtA.

Table 1

Oligonucleotide primers used for *cdtA*-His, *cdtB*-His and *cdtC*-His cloning.

Name	Sequence*a*
cdtA-F	5′-CCCAC<u>CCATGG</u>CTCCGAGGAGAGGTACAATGAAAAAG-3′
cdtA-R	5′-TTA<u>GGATCC</u>**GTGGTGGTGGTGGTGGTG**ATTAACCGCTGT-3′
cdtB-F	5′-GCCGCCGCGGTT<u>CCATGG</u>AATGGGTAAAGC-3′
cdtB-R	5′-TTA<u>GGATCC</u>**GTGGTGGTGGTGGTGGTG**GCGATCATGAA-3′
cdtC-F	5′-TCCCGCCGGTTTTGT<u>CCATGG</u>TCGCTAAGGAGAATAC-3′
cdtC-R	5′-AAT<u>GGATCC</u>**GTGGTGGTGGTGGTGGTG**GCTACCCTGATT-3′

*a*Underlined bases mark the NcoI and BamHI restriction endonuclease recognition sites. Bases in bold type mark the codons for the His-tag.

Table 2

Plasmids used in this study.

Plasmid	Features	Source or reference
pCDT1	*cdt* locus from *A. actinomycetemcomitans* cloned in *E. coli*	Mayer *et al.* (1999)
pJDA9	*cdtA* gene containing 6 His codons at 3′-end	This study
pJDB7	*cdtB* gene containing 6 His codons at 3′-end	This study
pJDC2	*cdtC* gene containing 6 His codons at 3′-end	This study
pMUT65cdtA	CdtA^{C196S}	This study
pMUT81cdtA	CdtA^{S165N}	This study
pMUT99cdtA	CdtA^{T88T}	This study
pMUT112cdtA	CdtA^{F43S}	This study
pMUT121cdtA	CdtA^{T41A}	This study
pMUT221cdtA	CdtA^{C178W}	This study
pMUT239cdtA	CdtA^{P56Q}	This study
pMUT241cdtA	CdtA^{T219A}	This study
pMUT41cdtC	CdtC	This study
pET15b	Cloning vector	Novagen

Table 3

Summary of point mutations.

Mutant designation	Nucleotide change	Amino acid change	Position in *H. ducreyi* CdtA	Comments
mutA62	A54C	None	—	
mutA65	A198T/T589A	None/C197S	C198	
mutA79	G348A	None	—	
mutA81	T452A	S165N	S166	
mutA99	T264A	None	—	
mutA112	T128C/A246G	F43S/none	P43	
mutA121	A121G	T41A	I41	
mutA183	G177A	None	—	
mutA221	T534G	C178W	C179	
mutA238	C142T	None	—	
mutA239	C166A	P56Q	S56	
mutA241	A655G	T219A	T220	
mutA249	A655G	T219A	T220	Same as mutA241
mutC41	A146T/A261G/	Q49L/none	NA	
	T430C/A517T/	None/S173C		

NA, not applicable.

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Table 4

Cell cycle analysis of CHO cells treated with reconstituted mutant holotoxin.

Form of CdtA	Diploid G1 (2n) (%)	Diploid G2 (4n) (%)	Diploid S (%)	Coefficient of variance (%)
none	75.35	5.93	18.72	2.71
CdtA-H	15.42	66.00	18.59	4.18
mutA65	4.79	70.85	24.37	9.87
mutA81	36.44	43.91	19.65	3.34
mutA99	8.71	78.14	13.16	5.04
mutA112	9.81	79.11	11.08	4.22
mutA121	73.74	9.92	16.34	3.32
mutA221	68.04	14.86	17.10	3.73
mutA239	4.90	79.28	15.81	5.99
mutA241	7.97	79.25	12.79	4.32
mutC41	66.03	15.43	18.54	3.78