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Assembly and function of a bacterial genotoxin

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The tripartite cytolytic distending toxoid (CDT) induces cell cycle arrest and apoptosis in eukaryotic cells2. The subunits CdtA and CdtC associate with the nuclease CdtB to form a holotoxin that translocates CdtB into the host cell, where it acts as a genotoxin by creating DNA lesions2–4. Here we show that the crystal structure of the holotoxin from Haemophilus ducreyi reveals that CDT consists of an enzyme of the DNase-I family, bound to two Cdt-Cdt-Cdt domains. CdtA, CdtB and CdtC form a ternary complex with three interdependent molecular interfaces, characterized by globular, as well as extensive non-globular, interactions. The lectin subunits form a deeply grooved, highly aromatic surface that we show to be critical for toxicity. The holotoxin possesses a steric block of the CdtB active site by means of a non-globular extension of the CdtC subunit, and we identify putative DNA binding residues in CdtB that are essential for toxin activity.

Many Gram-negative pathogens, such as Escherichia coli, Shigella dysenteriae, Actinobacillus actinomycetemcomitans, Campylobacter spp., Helicobacter spp., Salmonella typhi and H. ducreyi, possess a related toxic activity that causes cell cycle arrest and subsequent cellular distension in epithelial cells, and a rapid death by apoptosis in many lymphocytes4–6. This toxic activity is associated with the products of an operon that encodes three proteins: CdtA, CdtB and CdtC (refs 2–4). CdtB shares conserved residues with the active sites of DNase I-like nucleases3–5 and demonstrates nuclelease activity in vitro and in vivo6,7,8,9,21. CdtA and CdtC are required to form a tripartite complex with CdtB that delivers the CdtB subunit into cells, leading many eukaryotic cell types to activate DNA damage checkpoints5,6,8,14–21. Taken together, these facts suggest that this toxin may have carcinogenic properties3,4,10.

The crystal structure of the holotoxin from H. ducreyi reveals that CDT is a ternary complex with three extensive globular protein–protein interfaces (buring a total of over 6,300 Å2 of surface area), between CdtA–CdtB, CdtA–CdtC and CdtB–CdtC (Fig. 1a). In addition, CdtA and CdtC possess amino and carboxy termini that project as non-globular polypeptides (Figs 1 and 2) and interact extensively with each other and with CdtB (altogether burying another 4,400 Å2 of surface area), thereby contributing to an overall massive and interdependent surface area of interaction (over 10,700 Å2 for the entire holotoxin, with relative molecular mass 70,000 (M, 70k)).

CdtA and CdtC are both lectin-type structures, homologous to the B-chain repeats of the plant toxin, ricin22, consisting of a pseudo three-fold symmetric trefoil of 12 β-strands, arranged in three separate β-sheets. CdtA and CdtC align to the first repeat of the ricin B-chain with root mean square (r.m.s.) deviations of 2.3 Å and 3.7 Å over 119 and 108 amino acids, respectively (with 16% sequence identity between ricin and CdtA from a structural alignment)23. CdtA and CdtC bind at one end of CdtB, contacting each other (Fig. 1) in a manner reminiscent of the repeat arrangement in the ricin B-chain and its interaction with the active enzymatic subunit of this toxin23, although CdtA and CdtC cannot be superimposed as a rigid unit with the ricin repeats.

CdtA and CdtC together contribute towards the formation of two notable surface elements, both located on the face of the holotoxin that is directed away from CdtB. The first is a large aromatic cluster consisting of eight bulky side-chains, most of which are well conserved in CDT homologues (Fig. 1b). The second is a long and deep groove, or crevice, (approximately 20 × 10 Å long and 5–8 Å deep) that is formed from structural components of both proteins (Figs 1c and 2a).

Mutagenesis of the aromatic patch renders the holotoxin inactive in cellular assays, although the ternary complex stability is unimpaired (Fig. 3). Previous reports have shown that CdtA and CdtC are each able to bind to the surface of cells14–23, although the data for CdtC are conflicting2. Our structure and mutagenesis strongly support the idea that CdtA (through the aromatic patch) has an important role in the activity of this toxin, presumably through cell surface binding. Because CdtA and CdtC both contribute to the formation of the deep groove, the function of this structural element might be severely impaired when one of the components is absent, and may explain why full toxicity is only achieved when both components are together14–24. The CdtA N-terminal extension (discussed below) forms its crystal-packing contacts by interacting with this groove and inserting itself deeply into it (Fig. 2a). This raises the possibility that the CdtA N-terminus in the crystals may be mimicking a peptide from a cell surface receptor for CDT, implicating the groove as a potential peptid-binding cleft.

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The close interplay of CdtA and CdtC in the formation of the groove and aromatic patch, and the similarity in their positioning with the two lectin repeats in the ricin B-chain, suggest that these two components of CDT work together for a related function. This function is likely to be receptor binding to initiate endocytosis of the complex, and strongly supports the hypothesis that the CdtA–CdtC sub-complex can be viewed as the ‘B’ or ‘binding’ element of a two-component AB toxin, as had been proposed from previous biochemical studies. CDT would therefore be an AB₂ toxin, with CdtB component AB toxin, as had been proposed from previous biochemical studies. CDT would therefore be an AB₂ toxin, with CdtB taking the role of the active, or ‘A’, subunit.

CdtA and CdtC each have extended, non-globular polypeptides at their N and C termini, and in both proteins these regions interact with other elements of the holotoxin to cement the ternary complex together. The N terminus of the crystallized construct of CdtA extends to residue 18, and amino acids 18–56 are disordered. The non-globular extension from the CdtA N terminus is highly unusual, as amino acids 56–67 make only minor contacts to any elements of the holotoxin in the crystal asymmetric unit; instead they extend distally to make extensive crystal-packing contacts with a symmetry-related complex (Figs 1 and 2a). This is likely to be a crystal-packing artefact, as the CDT complex shows no signs of oligomerization by gel-filtration chromatography (Fig. 3a). It is probable, therefore, that residues 18–67 are normally disordered in the complex, but a fortuitous crystal-packing arrangement allowed for the region between 56 and 67 to become ordered. The amino acids 67–75 of this extended polypeptide, however, do contact CdtB and CdtC in the appropriate complex in the crystal, and therefore represent a true structural interaction. H. ducreyi CdtA is smaller than CdtA proteins from most other bacterial species, and CdtA is the most highly conserved of the three holotoxin components and shows weak sequence similarity to proteins of the DNase-I family (approximately 12% identity based on a structural alignment). The crystal structure reveals that CdtB adopts the canonical, four-layered fold for this family of enzymes, consisting of a central 12-stranded β-sandwich packed between outer α-helices and loops on each side of the sandwich, DNase I, HAP1 (human DNA repair endonuclease), and ExoIII (exonuclease III of E. coli) align with CdtB over the core fold with r.m.s. deviations in Ca positions of 3.3 Å, 3.3 Å and 2.9 Å, over 209, 198 and 197 residues, respectively. Overall, CdtB is most structurally similar to the DNase-I fold (as judged by the Z score).

In common with DNase I, CdtB possesses two absolutely conserved histidine residues (CdtB His 160 and His 274—Fig. 4a). These are coloured by degree of conservation between CdtA homologues, as indicated. The patch is at the top of the molecule, and the orientation of the figure is similar to CdtA in (a), rotated by 180° about a vertical axis. Surface groove and electrostatics of the CdtA–CdtC ‘top’ portion of the holotoxin (CdtB is beneath or ‘behind’ the surface shown, and the orientation of the holotoxin is like that in (a), rotated 90° about a horizontal axis).
putative catalytic histidines of CdtB are critical for toxin activity\(^9,10\) and superimpose almost perfectly with the active site histidines in structural alignments with DNase I (Fig. 4a). Only one of the catalytic histidines (CdtB His 274) hydrogen bonds with a nearby carboxylate group (CdtB Asp 238), as is observed in DNase I; an interaction that has been postulated to be important due to its raising of the histidine pK\(_a\) value during phosphodiester cleavage and for correctly orienting the catalytic residues 27–29. His\(_{160}\) (equivalent to DNase I His 134) does not have such a bonding partner, because Val\(_{118}\) of CdtB occupies the equivalent position of DNase I Glu\(_78\) (Fig. 4a).

Because mutation of these partner aspartic or glutamic acid residues in DNase I significantly lowers the activity of this enzyme\(^29,30\), it could be hypothesized that the very low DNase activity observed for CdtB in vitro (0.01% the activity of bovine DNase I)\(^{11,12}\) may be due to the presence of a valine (Val\(_{118}\)), instead of glutamate, at this position. In vitro, plasmid relaxation assays of wild type and a Val\(_{118}\)Glu mutant, however, show that the mutant is inactive in the conversion of supercoiled to relaxed or linear forms (Fig. 4c). Supporting the in vitro results, the Val\(_{118}\)Glu mutation rendered the assembled holotoxin completely inactive in a cellular toxicity assay (Fig. 4d), despite the fact that the ternary complex assembled in vitro indistinguishably from the wild type (data not shown). These results indicate that this apparently simple reversion (to glutamate) cannot be tolerated in CdtB.

Three highly conserved residues in DNase I, that make direct contacts to substrate DNA, have nearly identical counterparts in CdtB (Fig. 4a, b). Arg\(_{111}\) and Asn\(_{170}\) of DNase I have similar three-dimensional positions to CdtB Arg\(_{144}\) and Asn\(_{201}\) (two absolutely conserved residues in the CdtB subunit). A third DNA contact of DNase I, Arg\(_{41}\), also has a three-dimensionally equivalent residue from CdtB, Arg\(_{117}\). Although the Ca positions of DNA, positioned by aligning the DNA–DNase I complex with CdtB, is drawn with the phosphodiester backbone in yellow and the bases in light red, with the atoms of carbon, nitrogen and oxygen coloured yellow, blue and red, respectively.

Figure 2 Two non-globular extensions in the holotoxin. a, Surface representation (grey) of the crystallographic symmetry-related interaction between the groove and the N-terminal tail of CdtA. The symmetry is in a similar orientation to Fig. 1a, and is shown partially transparent. The symmetry-related holotoxin subunits are denoted by an asterisk. b, The molecular surfaces of CdtB and CdtC are shown in grey and green, respectively. DNA, positioned by aligning the DNA–DNase I complex with CdtB, is drawn with the phosphodiester backbone in yellow and the bases in light red, with the atoms of carbon, nitrogen and oxygen coloured yellow, blue and red, respectively.
Arg 41 (of DNase I) and Arg 117 (of CdtB) are only 2.0 Å apart and the side-chain atoms align closely (Fig. 4b), this arginine from CdtB is not located on the equivalent secondary structural element in the protein fold, but is situated on an adjacent β-strand, nearly 50 residues subsequent to the sequence position in CdtB that aligns with Arg 41 of DNase I. Other contacts between DNase I and DNA do not seem to have simple correlates in CdtB. When the three putative DNA-contacting residues are mutated, there is a complete loss of activity in cellular toxicity assays, but there are no defects in complex assembly, stability or solubility (Fig. 4d and data not shown).

In vitro nuclease assays, against plasmid, show that the mutation of these residues diminishes activity, but, unlike corresponding mutations in DNase I, does not abolish it (Fig. 4c). These results provide an important step towards a molecular understanding of this unique and potent toxin, as well a template for probing the function and cellular effects of CDT intoxication. In addition, the molecular details of the CdtB active site, the identification of likely substrate contacting residues, and the discovery of functionally important surfaces on the lectin domains, provide targets for therapeutic design to inactivate this toxin.

**Methods**

**Reconstitution and purification of the *H. ducreyi* holotoxin**

*H. ducreyi* CdtA(18–223), CdtB(23–283) and CdtC(21–186) were cloned by PCR (polymerase chain reaction) from genomic DNA (American Type Culture Collection number 700724D) as N-terminal hexahistidine fusion proteins in *E. coli* such that the predicted N-terminal secretion signals were removed. The three subunits were expressed separately and purified under denaturing conditions (8 M urea, 10 mM Tris pH 8.0, 0.1 M Na phosphate) using nickel chelating affinity resin. Following co-refolding by dialysis at 4°C (with total protein concentration under 0.1 mg ml⁻¹) into a native buffer consisting of 20 mM HEPES (N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid]) pH 7.5, 200 mM NaCl, 2.5 mM dithiothreitol (DTT), 5% glycerol and 2 mM ethylenediaminetetraacetic acid (EDTA), the proteins were separated from the affinity tag by site-specific proteolytic cleavage, and the holotoxin further purified by cation-exchange and gel-filtration chromatography.

**Crystallization and structural determination**

For crystallization, the purified preparation of the *H. ducreyi* holotoxin was concentrated by ultrafiltration to 10 mg ml⁻¹ in a buffer containing 20 mM HEPES pH 7.5, 200 mM NaCl and 2.5 mM DTT. Crystals of the holotoxin were grown at 23°C by vapour diffusion using hanging drops formed from mixing a 1:1 volume ratio of 10 mg ml⁻¹ complex with an equilibration buffer consisting of 15–25% polyethylene glycol monomethylether (PME-M, 5K) 25–30% glycerol and 0.1 M imidazole, pH 6.5, supplemented with 2 mM DTT. Crystals formed in the space group P2₁2₁2₁, with cell dimensions a = 71.7 Å,
b = 75.8 Å and c = 121.8 Å, with one holotoxin complex in the asymmetric unit. For cryoprotection, crystals were transferred directly into a buffer with PME (M, 5K), concentration 3–10% higher than the conditions in which the crystals grew, with 20% glycerol, supplemented with 1 M sodium bromide, and flash-cooled immediately afterward to ~160 °C (total transfer/soak time between 20–30 s). Phases were determined by single-wavelength anomalous diffraction from the scattering of 26 partially ordered bromide ions introduced from the cryo-buffer. Crystallographic methods and statistics can be found in Supplementary Information. The final model was refined to 2.0 Å and has R/Rw values of 18.2% and 21.4%, respectively. 90% of the residues fall into the most favourable region of the Ramachandran plot without outliers. The N-terminal 38 residues (18–56) of the crystallized construct of CdtA are not visible in the electron density, nor are residues 183–185 of CdtC (internal loops). Four residues at the N terminus and eight at the C terminus of CdtC are also not modelled, owing to disorder.

**Mutagenesis**

The amino-acid substitutions were introduced into cdB genes by in vitro site-directed mutagenesis using oligonucleotide primer pairs containing the appropriate base changes. The amplification of the mutant plasmids was generated by PCR and cloned as N-terminal hexahistidine fusion protein into the same vector as wild-type gene. All mutants created were verified by DNA sequencing.

**Cell cycle analysis**

Cell cycle progression of HeLa cells treated with CDT holotoxin was analysed by flow cytometry. HeLa cells (2 × 10^6 cells) were treated with indicated concentrations of wild-type or mutant CDT holotoxin for 3 h at 37 °C, 5% CO₂, and were subsequently washed of toxin and maintained in culture medium. 48 hours after treatment, cells were collected and fixed by addition of cold 70% ethanol during continuous vortexing, and left for 4 h at 16°C. The cells were then stained for 2 h at 23 °C with propidium iodide solution (25 μg ml⁻¹ propidium iodide, 100 U ml⁻¹ DNase-free RNase A, 0.04% TritonX-100 in PBS). Relative DNA content was measured by flow cytometry with FACSCalibur flow cytometer (Beckton Dickinson).

**Plasmid-digestion assay**

The DNase activity of CdtB was tested by a plasmid-digestion reaction as previously described. Wild-type and mutant CdtB proteins used in this assay were purified using the same protocol described above for the CDT holotoxin. The reaction contained 20 mM HEPES pH 7.5, 150 mM NaCl, 5 mM MgCl₂, 5 mM CaCl₂, supercoiled pUC19 plasmid (2.5 μg) as substrate, and indicated concentrations of wild-type or mutant CdtB (1, 2, or 9 μg), or CDT holotoxin (3, 9, and 27 μg). The reaction was incubated for 5 h at 37 °C, and quenched by addition of 10 mM EDTA. Supercoiled, linear and relaxed plasmid forms were separated by agarose gel electrophoresis (0.8%), stained with ethidium bromide, and photographed using Gel Doc 2000 Documentation system (Bio-Rad).

**Supplementary Information**

accompanies the paper on www.nature.com/nature.

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**Authors’ contributions**

DN—cloning of wild-type and mutant CDT holotoxin and CdtB, protein purification, activity assays, and crystallography, and YH—mutant CdtA cloning and purification of mutant CdtA containing holotoxin.

**Competing interests statement**

The authors declare that they have no competing financial interests.

Correspondence and requests for materials should be addressed to C.E.S. (stebbins@rockefeller.edu). Coordinates of the structure have been deposited in the Protein Data Bank under the accession number 1SR4.

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**Human meiotic recombinase Dmc1 promotes ATP-dependent homologous DNA strand exchange**

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Homologous recombination is crucial for the repair of DNA breaks and maintenance of genome stability. In Escherichia coli, homologous recombination is dependent on the RecA protein. In the presence of ATP, RecA mediates the homologous DNA pairing and strand exchange reaction that links recomb