Filamentation induced by cAMP (Fic) domain proteins have been shown to catalyze the transfer of the AMP moiety from ATP onto a protein target. This type of post-translational modification was recently shown to play a crucial role in pathogenicity mediated by two bacterial virulence factors. Herein we characterize a novel Fic domain protein that we identified from the human pathogen Clostridium difficile. The crystal structure shows that the protein adopts a classical all-helical Fic fold, which belongs to class II of Fic domain proteins characterized by an intrinsic N-terminal autoinhibitory α-helix. A conserved glutamate residue in the inhibitory helix motif was previously shown in other Fic domain proteins to prevent proper binding of the ATP γ-phosphate. However, here we demonstrate that both ATP binding and autoadenylation activity of the C. difficile Fic domain protein are independent of the inhibitory motif. In support of this, the crystal structure of a mutant of this Fic protein in complex with ATP reveals that the γ-phosphate adopts a conformation unique among Fic domains that seems to override the effect of the inhibitory helix. These results provide important structural insight into the adenylylation reaction mechanism catalyzed by Fic domains. Our findings reveal the presence of a class II Fic domain protein in the human pathogen C. difficile that is not regulated by autoinhibition and challenge the current dogma that all class I-III Fic domain proteins are inhibited by the inhibitory α-helix.

Clostridium difficile is a Gram-positive, anaerobic, and spore-forming pathogen recognized as the leading cause of healthcare-associated diarrhea (1–3). The incidence and severity of these infections have been increasing in the past decade, and treatment is difficult and expensive. The virulence is primarily attributed to increased expression of two large toxins, TcdA and TcdB, which have both been shown to inactivate Rho GTPases in host cells by glycosylation of a threonine residue in the switch I region (4, 5). However, there are contrasting reports on the pathological importance of these toxins. In one hamster study, TcdB was found to be essential for C. difficile virulence (6). Another group found that both the A′B′- and A−B+ strains are able to cause disease in hamsters (7), whereas a third study concluded that TcdA was the major determinant for virulence (8). Adding to the confusion, a highly virulent strain was recently identified where the pathogenic phenotype could not be attributed to increased toxin production. Instead, this strain was shown to hold additional laterally acquired DNA containing genes of hypothetical function (9). Therefore, increased virulence is also likely associated with accessory virulence factors, which have not yet been described.

Recently, two bacterial virulence factors, LbpA from H. pylori and VopS from Vibrio parahaemolyticus, were also discovered to inactivate host cell Rho GTPases by generating a phosphodiester bond with the AMP moiety on either a conserved tyrosine or threonine residue in the switch I loop (10, 11). This modification is known as adenylylation or AMPylation and is catalyzed by the filamentation induced by cAMP (Fic) domain. Fic domains have been identified across all domains of life and shown to be responsible for a multitude of functions varying from bacterial virulence effective in host cells to regulation of eukaryotic intracellular proteins (12). SidM/DrrA from Legionella pneumophila was shown to adenylylate Rab1 GTPases and manipulate host membrane trafficking (13). Fic-mediated adenylylation is also involved in eukaryotic cell signaling (14), and very recently the human huntingtin yeast interacting protein E (HYPE) and a homologous Drosophila Fic protein were shown to reversibly adenylylate the molecular chaperone BiP in the endoplasmic reticulum (15, 16). Additionally, the toxin-antitoxin Fic protein complex VbTA from Bar- toronella schoenbuchensis was shown to control growth of other

A Novel Fic (Filamentation Induced by cAMP) Protein from Clostridium difficile Reveals an Inhibitory Motif-independent Adenylylation/AMPylation Mechanism*5

Received for publication, November 19, 2015, and in revised form, April 8, 2016. Published, JBC Papers in Press, April 13, 2016, DOI 10.1074/jbc.M115.705491

Emil Dedic†1, Husam Alsarraf†, Ditte Hededam Welner†, Ole Østergaard†, Ole I. Klychnikov§, Paul J. Hensbergen†, Jeroen Corver†, Hans C. van Leeuwen‡, and René Jørgensen†‡

From the Departments of †Microbiology and Infection Control and §Autoimmunology and Biomarkers, Statens Serum Institut, DK-2300 Copenhagen S, Denmark and the ‡Center for Proteomics and Metabolomics, and †Department of Medical Microbiology, Section Experimental Bacteriology, Leiden University Medical Center, 2300RC Leiden, The Netherlands

*This work was supported by Det Frie Forskningsråd Sapere Aude Starting Grant 11-104831/Medical Sciences from the Danish Council for Independent Research. Access to synchrotron beam time was made possible by support from DANSCATT. The authors declare that they have no conflicts of interest with the contents of this article.

†This article contains supplemental Fig. 1.
The atomic coordinates and structure factors (codes 4X2C, 4X2D, and 4X2E) have been deposited in the Protein Data Bank (http://wwpdb.org/).

+ Present address: Inst. of Molecular Biology and Biophysics, Dept. of Biology, Eidgenössische Technische Hochschule (ETH), 8093 Zürich, Switzerland.

§To whom correspondence should be addressed: Dept. of Microbiology and Infection Control, Statens Serum Institut, Artillerivej 5, DK-2300 Copenhagen S, Denmark. Tel.: 45-3268-3268; Fax: 45-8612-3178; E-mail: renj@ssi.dk.

3 The abbreviations used are: Fic, filamentation induced by cAMP; α-inh, inhibitory α-helix; HYPE, huntingtin yeast interacting protein E; AU, asymmetric unit; SEC, size exclusion chromatography; DSF, differential scanning fluorimetry; EOM, ensemble optimization method; SAXS, small angle x-ray scattering; SE/AA, S31A/Einh35A; Cd, C. difficile; Nm, N. meningitides; Bt, B. thetaiotaomicron; Hp, H. pylori.
bacteria by inactivating DNA gyrase and topoisomerase IV via adenylylation (17).

In the absence of a protein target, Fic domain proteins are known to catalyze autoadenylylation where AMP is transferred to a residue in proximity of the active site (18–22). The biological significance of this self-labeling is unclear but has been proposed to either represent a reaction intermediate in the phosphoryl transfer to a target protein or function in regulation of enzyme activity (22, 23). Some Fic domains are capable of catalyzing post-translational modifications other than adenylylation. The plant pathogen AvrAC from *Xanthomonas campes-tris* suppresses the host immune response by transferring UMP from UTP to the host kinases BIK1 and receptor-interacting protein kinase (RIPK) (20), whereas IbpA has affinity for both GTP and ATP (24). In addition, the cofactor specificity is not restricted to triphosphate nucleotides as the *Legionella* effector AnkX utilizes CDP-choline to target Rab1 GTPase with a phosphocholine modification (25). Finally, the more distantly related bacteriophage toxin Doc inhibits bacterial translation by phosphorylating the translation elongation factor EF-Tu (26).

Fic domains are defined by the highly conserved active site Fic motif HXX(F/D/E)(A/G)N(G/K)XXR but otherwise share very little sequence identity. Also, the Fic domains can be found either as single domain proteins or as individual domains in multidomain proteins. A typical Fic domain consists of a structural core of eight α-helices described as a six-helix up-and-down bundle (α1−α5 and α’1) with helices α6−α7 lying roughly perpendicular to the bundle (18). The highly conserved and essential histidine of the Fic motif is the catalytic residue believed to deprotonate the attacking hydroxyl group of the target protein (19, 21, 27). Fic proteins also include the flap, either a β-hairpin or a loop, bridging helices α2−α3, proposed to mediate substrate binding (18, 19, 23). Recently, it was shown that adenylylation activity of some Fic domains is controlled by a conserved mechanism of ATP binding site obstruction involving an intrinsic inhibitory α-helix (αinh), containing a conserved (S/T)XXEinh(G/N) inhibitory motif (21). The mechanism of inhibition is divided into three different classes depending on whether αinh is provided intermolecularly by an interacting antitoxin (class I) or positioned intramolecularly in the Fic protein either as an N-terminal (class II) or a C-terminal (class III) α-helix. The conserved glutamate in this inhibitory motif, Gluoinh, has been found to cause a steric hindrance of proper positioning of the ATP γ-phosphate (21, 28). This auto-inhibition was further supported by substitutions of Gluinh with either alanine or glycine, which resulted in increased levels of autoadenylylation as well as target adenylylation in vitro (15, 16, 21, 28).

We have previously identified a novel Fic domain protein, CdFic, from the Gram-positive human pathogen *C. difficile* (29). To elucidate the molecular function of CdFic, we have structurally characterized the purified protein both with and without ATP bound in the active site. We show that CdFic forms a stable dimer despite an unusually small interaction interface. Furthermore, ATP binding triggers a transition of the flap from a disordered/open to an ordered/closed conformation. Although the overall structural fold is similar to other Fic domains, we show that CdFic binds ATP as well as catalyzes autoadenylylation independently of the inhibitory motif.

**Experimental Procedures**

**Cloning, Purification, and Crystallization**—The gene (CDR20291_0569) from *C. difficile* strain R20291 (accession number YP_003217073) encoding CdFic and the CdFicSE/AA mutant (containing S31A and Einh35A substitutions in the inhibitory motif) with a C-terminal His6 tag was cloned, expressed, and purified as described previously (29). Codons 37 (AGT), 38 (ACT), 57 (CAT), 163 (GAT), and 200 (AGA) encoding serine, threonine, histidine, and arginine, respectively, were substituted by alanine codons (GCT) using QuikChange site-directed mutagenesis according to the manufacturer’s instructions (Agilent Technologies). The *Escherichia coli* expression plasmids pFVS0015 and pFVS0059, encoding NmFic and the NmFicE186G mutant, respectively, from *Neisseria meningitides* were kindly provided by Schirmer and co-workers (21) at Biozentrum, University of Basel. After induction in *E. coli* BL-21 cells, NmFic and NmFicE186G were purified by nickel affinity chromatography using standard procedures. Crystallization experiments were performed using sitting drop vapor diffusion at room temperature. CdFic crystallized in a 2-μl drop with a 1:1 ratio of protein:reservoir solution containing 25% (v/w) PEG-8000, 0.2 M MgCl2, and 0.1 M Hepes, pH 7.5. CdFicSE/AA crystallized in a 4-μl drop with a 3:1 ratio of protein:reservoir solution containing 25% (v/w) PEG-3350, 0.2 M MgCl2, and 0.1 M Hepes, pH 7.5. CdFicSE/AA-ATP crystals were obtained by gradually soaking drops of CdFicSE/AA crystals with increasing amounts of a 10 mM stock solution of ATP dissolved in reservoir buffer and incubated at room temperature for 20 min. The crystals appeared within 24 h with use of freshly prepared streak seeds from initial smaller crystals using the Seed Bead (Hampton Research). Crystals were flash cooled in liquid N2 after a cryoprotective solution containing reservoir solution and 20% (v/v) glycerol was gradually added to the drop.

**Data Collection, Reduction, and Structure Solution**—X-ray diffraction data on CdFic crystals were collected to 3.1-Å resolution at beamline ID23 at the European Synchrotron Radiation Facility (λ = 0.979 Å, 100 K). CdFicSE/AA data were collected to 1.8-Å resolution at beamline 1911-2 at the MAX-II Laboratory in Lund, Sweden (λ = 1.038 Å, 100 K). Data on the CdFicSE/AA-ATP complex were collected to 2.5-Å resolution at European Synchrotron Radiation Facility beamline ID29 (λ = 0.900 Å, 100 K). All data sets were integrated and scaled by XDS (30). After integration and scaling, all three structures were solved by molecular replacement using the CCP4i module Phaser (31) with the previously solved structure of the signaling protein BtFic from *Bacteroides thetaiotaomicron* (Protein Data Bank code 3CU) having 26% sequence identity to CdFic as a search model. Initial rigid body and restrained refinement was carried out using REFMAC5 (32) before iteratively rebuilding the structure using Coot (33) and finally refining it in Phenix (34) including Translation, Liberation, Screw groups (35). For the CdFic and the CdFicSE/AA-ATP structures, NCS restraints were introduced during the refinement steps in Phenix. The atomic coordinates are deposited at the Protein Data Bank. All the
Crystal Structure of a Fic Protein from *C. difficile*

Structural figures were prepared using PyMOL (36). The buried surface area of the dimer was calculated using PDBBind (37).

Small Angle X-ray Scattering (SAXS)—All SAXS synchrotron radiation data were collected at beamline I1911-4 at the MAX-II Laboratory as 4 × 30-s exposures of a 20–30-μl sample, and scattering profiles were compared to detect radiation damage. The buffer consisted of 20 mM Tris, pH 7.5, 100 mM NaCl, 5 mM MgCl₂, and 5 mM 2-mercaptoethanol. Data were collected at 0.91-Å wavelength at 10 °C using a hybrid pixel Pilatus 1M detector (Dectris). To detect concentration-dependent interparticle effects, measurements were collected at multiple protein concentrations in the range of 0.65–5 mg/ml. The data collected at 2.6 mg/ml were used for further analysis as concentrations above this exhibited minor interparticle effects. Back-scatter data were used for further analysis as concentrations above this exhibited minor interparticle effects. Back-scatter data were used for further analysis as concentrations above this exhibited minor interparticle effects.

The flow rate was 200 nl/min; the mobile phases consisted of 0.1% acetic acid in solvent A (water) and 0.1% acetic acid in solvent B (acetonitrile). MS data were recorded in parallel in a data-dependent mode, fragmenting the five most abundant ions (charge state, 2 or higher) by collision-induced dissociation in the LTQ ion trap. Mass spectra were acquired recording full scan spectra (mass/charge (m/z)). Desalted peptides were loaded on an Acclaim PepMap C₁₈ precolumn (300 × 5 × 5 mm; Dionex) and separated using an Acclaim PepMap100 C₁₈ analytical column (75 × 150 × 3 μm, 3-μm particle size; Dionex) by a 75-min gradient controlled by a Dionex Ultimate 3000 system connected to an LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific) equipped with a nanoelectrospray source (Proxeon, Odense, Denmark).

The flow rate was 200 nl/min; the mobile phases consisted of solvent A (2% (v/v) acetonitrile and 0.1% formic acid) and solvent B (95% (v/v) acetonitrile and 0.1% (v/v) formic acid). The gradient went from 0 to 45% solvent B in 65 min followed by 10 min with 100% solvent B; then data acquisition was stopped, and the column was re-equilibrated with solvent A. MS data were acquired recording full scan spectra (mass/charge (m/z), 250–1,800) in the Orbitrap with 60,000 resolution at 400 m/z. MS/MS data were recorded in parallel in a data-dependent mode, fragmenting the five most abundant ions (charge state, +2 or higher) by collision-induced dissociation in the LTQ ion trap at 35% collision energy. MS/MS spectra were recorded using dynamic exclusion (30 s) to minimize repeated fragmentation of the same peptides and analyzed using MaxQuant version 1.4.12.2 (52) for peptide and protein identification using the built-in Andromeda search engine (53). The following settings were used: His₆-CdFic⁺⁵⁺ sequence (29) with search against the built-in Andromeda search engine (53).
Crystal Structure of a Fic Protein from C. difficile

Crystallographic data and refinement statistics

| Data collection | CdFicSE/AA | CdFicSE/AA-ATP | CdFic |
|-----------------|------------|----------------|-------|
| Radiation source | MAX-II I911-2 | ESRF ID29 | ESRF ID23 |
| Wavelength (Å)   | 1.038      | 0.900         | 0.873 |

Data processing

|                | CdFicSE/AA | CdFicSE/AA-ATP | CdFic |
|----------------|------------|----------------|-------|
| Space group    | P2,2,2,1   | C2             | C22,  |
| Protein mol/AU | 2          | 4              | 4     |
| Cell dimensions| a = 52.4 Å, b = 67.3 Å, c = 139.6 Å, \(a = \beta = \gamma = 90\)° | a = 158.1 Å, b = 60.1 Å, c = 124.8 Å, \(a = \beta = \gamma = 90\)° | a = 57.4 Å, b = 157.4 Å, c = 262.7 Å, \(a = \beta = \gamma = 90\)° |
| Resolution (Å) | 20.0–1.8 (1.9–1.8) | 20.0–2.5 (2.6–2.5) | 20.0–3.1 (3.2–3.1) |
| Completeness (%)| 98.6 (97.2) | 99.7 (100) | 99.9 (99.9) |
| CC(1/2) on test set | 99.9 (77.9) | 99.9 (57.9) | 99.6 (82.1) |
| Mean \(R_{merge}\) | 6.6 (6.5) | 6.9 (7.2) | 8.0 (8.4) |
| Multiplicity | 7.7 (95.7) | 8.2 (155.2) | 17.3 (114.0) |

Refinement

|                  | CdFicSE/AA | CdFicSE/AA-ATP | CdFic |
|------------------|------------|----------------|-------|
| Resolution (Å)   | 20.0–3.1   | 20.0–2.5       | 20.0–3.1 |
| Reflections/Å2    | 35,839     | 20,454         | 20,454 |
| Reflections/°     | 1,076      | 1,026          | 1,026 |
| \(R_{merge}\) / \(R_{free}\) | 18.4/21.8 | 20.4/23.3 | 23.3/28.6 |
| Protein atoms     | 3,470      | 6,743          | 6,561 |
| Water atoms       | 216        | 14             |       |
| Glycero/PO4/Hepes atoms | 50       |                 |       |
| ATP/Mg2+ atoms    | 32         |                |       |
| Overall B-factor (Å2) | 26.3     | 60.1           | 51.9  |
| r.m.s.d. bond lengths (Å) | 0.008    | 0.010          | 0.003 |
| r.m.s.d. bond angles (°) | 0.999   | 0.921          | 0.779 |

Ramachandran statistics (%)

|                  | CdFicSE/AA | CdFicSE/AA-ATP | CdFic |
|------------------|------------|----------------|-------|
| Favored          | 99.0       | 98.4           | 98.3  |
| Allowed          | 1.0        | 1.5            | 1.7   |
| Outliers         | 0          | 0.1            | 0     |
| Protein Data Bank code | 4X2C     | 4X2D           |       |

* Values in parenthesis are for the highest resolution shell.

* Percentage of correlation between intensities from random half-data sets (65).

\(R_{merge}^* = 1 - \frac{\sum_{hkl} \left| F_{hkl} - \langle F_{hkl} \rangle \right|^2}{\sum_{hkl} \left| F_{hkl} \right|^2}\). Redundancy-independent \(R\) factor calculated on intensities (66).

\(R = \frac{\sum_{h} \left| F_{h} \right| - \left| F_{calc} \right|}{\sum_{h} \left| F_{calc} \right|}\), where \(F_{calc}\) and \(F_{obs}\) are observed and calculated structure factor amplitudes, respectively.

* The \(R_{free}\) value was calculated with a random 3 or 5% subset of all reflections excluded from refinement.

common contaminant sequences enabled; variable modifications
adenylation (STY; +329.05 Da), heavy adenylylation
(STY; +344.07 Da), oxidation (Met), and acetyl (protein N-terminal);
fixed modification, carbamidomethyl (Cys); labels,
none; peptide false discovery rate, 1%; protein false discovery rate,
1%; match between runs, 1 min; keep low scoring version
of identified peptides, on; all other settings, left at the default
settings. After automatic identification of the adenylylated pep-
tides by MaxQuant, the corresponding MS/MS spectra were
subjected to manual inspection, and diagnostic ions of 348.1
(AMP) and 363.2 Da (\([^{13}C_{10}^{15}N_2]AMP\) were observed
as described previously (54) and further confirmed the adenylyla-
tion of the IAGSTFTTEALALLLDK peptide.

Results

Crystal Structure of CdFic—To investigate the structure and
function of the newly identified Fic protein from C. difficile
(29), we solved the x-ray crystal structures of CdFic as well as
the \(\alpha_{inh}\) mutant CdFicSE/AA. This double mutant was chosen
hoping that autoinhibition would be released and that \(\alpha_{inh}\)
would dissociate from the nucleotide binding pocket as seen
previously in the NmFicSE/AA mutant structure (21) and thus
result in a more active enzyme. Moreover, by soaking
CdFicSE/AA crystals with ATP, we also solved the structure of
CdFicSE/AA in complex with ATP and Mg2+. The maximum
resolution of the diffraction data were 3.1 Å for CdFic, 1.8 Å for
CdFicSE/AA, and 2.5 Å for the CdFicSE/AA-ATP complex with

final \(R_{free}\) values of 28.6, 21.8, and 23.3%, respectively. The three
structures were solved by molecular replacement using the
homologous BtFic from B. thetaiotaomicron as a search model
(Protein Data Bank code 3CUC). According to PROCHECK
(55), Ramachandran plots for the three structures show that all
residues are within the allowed region except for a single out-
lying residue in the CdFicSE/AA-ATP structure. The details of
the data collection and refinement are provided in Table 1. The
CdFicSE/AA structure contains two molecules in the asymmet-
ric unit (AU) and belongs to space group P2,2,2,1, whereas both
the CdFic (space group C22, 1) and the CdFicSE/AA-ATP (space
group C2) structures contain four molecules in the AU. In the
CdFicSE/AA structure, the ATP binding sites of both molecules
in the AU are blocked by a glutamate in the C terminus of a
symmetry-related molecule. Luckily, the crystallization condi-
tion for CdFicSE/AA also produced another crystal form con-
taining four molecules in the AU of which two of the molecules
have an unoccupied binding site. This was the only crystal form
in which we were able to soak ATP into the binding site. Simi-
larly, only two of the four molecules in the AU of the CdFic
crystal had unoccupied ATP binding sites, but attempts to soak
ATP into the binding site of these crystals were unsuccessful.

The overall conformation of the three structures is highly
isomorphous with a root mean square deviation of 0.49 Å
between the A chains of CdFic and CdFicSE/AA as calculated
by the SSM Superpose tool in Coot using 197 Cα atoms. The CdFic
structure reveals a classical α-helical Fic fold with stacking of three consecutive helix-loop-helix elements (Fig. 1, A and B). In the Fic core element, the Fic motif (residues 163–174) bridges helices α4–α5, whereas the flaps bridges helices α2–α3. CdFic contains three additional N-terminal helices as an extension to the Fic core of which the middle helix adopts a location deeply embedded in the Fic core that is analogous to an N-terminal α_{inh} (Fig. 1, A and B). This N-terminal α_{inh} places CdFic among the class II Fic domain proteins (21) and harbors the proposed inhibitory motif residues Ser-31/Glu_{inh}/35, which are situated in the C terminus of the α_{inh} helix. Structural alignment of CdFic with BtFic from B. thetaiatotaomicron, the human HYPE, the second Fic domain of Histophilus somni, IbpAFic2, and HpFic from Helicobacter pylori reveals a common arrangement of the Fic core domain consisting of helices α1–α7 (Fig. 1C).

Interestingly, in the CdFic^{SE/AA} structure, there is well defined electron density for the α_{inh} helix, and its position is equivalent to the corresponding helix in CdFic (Fig. 1D). Hence, replacing these two residues in the inhibitory motif does not destabilize the α_{inh} helix as seen for the corresponding SE/AA mutant of the class III NmFic protein (21). This is not surprising because the interaction surface between α_{inh} and the CdFic core consists mainly of hydrophobic residues (Fig. 1E), which likely stabilize the core of the protein. In support of this, attempts to express and purify a CdFic(Δ1–40) deletion mutant resulted in a highly insoluble protein, indicating that removal of the first two α-helices including α_{inh} dramatically alters the properties of the protein.

In both the CdFic and CdFic^{SE/AA} structures, the major part of the flap is missing from the electron density maps and is supposedly flexible (Fig. 1, A and D). However, in the ATP-bound structure, the entire flap is ordered and almost completely covers the adenine base, leaving only the three phosphates solvent-exposed (Fig. 1F). Only molecule A of the four chains in the AU contains the ATP cofactor in the binding site as symmetry-related molecules block the binding site of molecules C and D. Molecule B does have an unobstructed binding site; however, movements of the flap are possibly prevented by a symmetry-related molecule D, and loop closure by the flap is likely necessary to lock the ATP in the binding site. Conversely, the binding site of molecule A is surrounded by enough space to allow loop closure after ATP binding without disrupting any crystal contacts. Of all 10 molecules within the AU of the three different CdFic crystal structures, the entire flap is only visible in the molecule with ATP bound. This is unlikely to be a coincidence and strongly suggests that ATP binding triggers a transformation of the flexible flap from a disordered/open to an ordered/closed conformation. As the flaps in other Fic domain proteins structures without ATP are either well defined or only partially disordered (19, 23, 56), the flap in CdFic seems particularly flexible without ATP.

Non-obstructed ATP Conformation—The CdFic structure contains the classical Fic nucleotide binding features consisting of a hydrophobic adenosine binding pocket, the ribose-coordinating arginine, and the GNG anion hole of the Fic motif. In the nucleotide binding pocket of the CdFic^{SE/AA}-ATP structure, there is well defined electron density for an intact ATP molecule coordinating a Mg^{2+} ion (Fig. 2A). The adenosine is locked into a pocket formed among helix α4, α6, and the flap (Fig. 1F) where Val-122, Ile-124, Leu-161, Leu-203, and Ile-117 form a hydrophobic patch. Only a single hydrogen bond is formed between the surrounding residues (Asn-204) and the adenine base (Fig. 2B). As in other Fic Glu_{inh}/Gly mutant structures in complex with ATP, the ribose is primarily coordinated by hydrogen bonds to the conserved Fic motif Arg-174 and Tyr-199, which help tether the middle of the nucleotide (Fig. 2B) (21, 56). Also, the GNG anion hole and the catalytic Fic motif histidine are situated similarly to other Fic domains. Hence, the Fic motif in the ATP binding pocket is highly conserved and shows no structural features deviating from other ATP-binding Fic domain proteins. Despite this, the ATP phosphates in the CdFic^{SE/AA}-ATP structure are positioned significantly differently in comparison with those of other Fic Glu_{inh}/Gly mutant structures in complex with ATP or ATP analogues (Fig. 2C). In the CdFic^{SE/AA}-ATP structure, the γ-phosphate forms an elaborate network of hydrogen bonds to both GNG backbone amide nitrogens and to Arg-171 of the Fic motif (Fig. 2B). To our knowledge, only one other Fic structure, the VbhT-VbhA-ATP complex (Protein Data Bank code 3ZC7), has a γ-phosphate positioned similarly within the GNG anion hole. However, the substitution of Glu_{inh} with a glycine in VbhA allows the γ-phosphate to move out of the anion hole and into a position overlapping with the Glu_{inh} side chain (Protein Data Bank code 3ZCB). Instead, in all other E_{inh}/G Fic mutant structures in complex with ATP or ATP analogues published to date, the anion hole is responsible for coordinating the α- and β-phosphates with the asparagine in the Fic motif, forming a salt bridge to the α-phosphate (Fig. 2D) (21, 28, 56). In the CdFic^{SE/AA}-ATP structure, however, the α- and β-phosphates are pulled away from the anion hole toward the opposite side of the active side cleft, and the α-phosphate instead forms a salt bridge to Arg-200 positioned in helix α6 of the Fic core (Fig. 2E).

Structural alignment of known Fic structures shows that CdFic is the only Fic protein containing an arginine at this position as other Fic domain structures predominantly have hydrophobic residues at the structurally equivalent position (Fig. 2, D and F). The Arg-200 residue might therefore explain the unique orientation of the ATP phosphates in CdFic. The carboxyl side chain of Asp-167 in the Fic motif of CdFic^{SE/AA} helps coordinate a Mg^{2+} ion together with the ATP β- and γ-phosphates (Fig. 2, B and E). This type of coordination is also in contrast to other Fic-ATP structures where the Mg^{2+} ion is instead situated between the α- and β-phosphates (Fig. 2, C and D). The coordinated Mg^{2+} ion in CdFic, however, suggests that ATP binds in an adenylation-competent conformation. Furthermore, this arrangement of ATP phosphates in the CdFic^{SE/AA}-ATP structure implies that Glu_{inh} would not obstruct proper positioning of the γ-phosphate in wild type CdFic. A superposition of the CdFic and CdFic^{SE/AA}-ATP structures shows that Glu_{inh} in CdFic is positioned immediately below the γ-phosphate and that the position of the glutamate side chain is replaced by a water molecule in the CdFic^{SE/AA}-ATP structure that forms hydrogen bonds to both the γ-phosphate and Arg-174 (Fig. 2G).

CdFic Dimerization Interface and Active Site Closure—In all three CdFic structures, two molecules form the same type of
FIGURE 1. **CdFic crystal structures.** A, the crystal structure of CdFic. The Fic domain core is shown in green, the N-terminal (N-term.) extension including the α<sub>inh</sub> is in dark yellow, the C-terminal (C-term.) extension is in violet, the conserved Fic motif loop is in blue, the dimerization loop is in black, and the position of the disordered flap is schematically shown with red spheres. Secondary structures in all other panels are colored accordingly. The α<sub>inh</sub> residues Ser-31 and Glu<sub>inh</sub>-35 are represented with sticks and highlighted by arrows. B, topological diagram based on the CdFic structure. C, structural superposition of Fic crystal structures represented with secondary structure and shown as a schematic diagram with each box representing a helix (B. thetaiotaomicron; Protein Data Bank code 3CUC), HYPE (Homo sapiens; Protein Data Bank code 4U07), lbpAFic2 (H. somni; Protein Data Bank code 4ITR), and HpFic (H. pylori; Protein Data Bank code 2F6S). The position of α<sub>inh</sub> is labeled inh, and TPR is the tetratricopeptide repeat motif of HYPE. D, crystal structure of CdFic<sup>S31A/E35A</sup>. The inhibitory motif substitutions S31A and E<sub>inh</sub>35A are represented with sticks and highlighted by arrows. The electron density for α<sub>inh</sub> is shown in gray. E, the interaction surface between the α<sub>inh</sub> helix (dark yellow) and residues in the CdFic core (green) with the α<sub>inh</sub> surface envelope colored in gray. F, crystal structure of the CdFic<sup>S31A/E35A</sup>-ATP complex shown in two orientations related by a 90° rotation about the horizontal axis. ATP is represented with black, blue, red, and yellow sticks, and the ordered/closed flap is colored in red.
FIGURE 2. The ATP binding site. A, the CdFicSE/AA-ATP binding pocket. An unbiased Fo/Fc electron density omit map around ATP is contoured at 4 σ and shown in gray. An Fo/Fc electron density omit map around Mg²⁺ ion is shown in green and contoured at 6.5 σ to emphasize the strong Mg²⁺ peak. Mg²⁺ is shown in purple, and the Fic motif and the flap are shown in blue and red, respectively. B, schematic diagram of the CdFicSE/AA-ATP coordination as determined by LIGPLOT (63). C, superposition of ATP from CdFicSE/AA-ATP crystal structure and the structures of HYPEE234G-ATP (H. sapiens; Protein Data Bank code 4U07), NmFicE186G-AMPPNP (N. meningitides; Protein Data Bank code 3ZLM), SoFicE73G-AMPPNP (Shewanella oneidensis; Protein Data Bank code 3ZEC), and BsFic (VbhT-VbhAE24G-ATP) (B. schoenbuchensis; Protein Data Bank code 3ZCB). D, the ATP binding mode of Fic domain structures from C not including CdFicSE/AA. E, the ATP binding mode of CdFicSE/AA highlighting the salt bridge between Arg-200 and γ-phosphate shown in identical orientation as D. F, structurally based multiple sequence alignment of Fic domains. Fic domains with the indicated Protein Data Bank accession codes were aligned using the program Strap (64), and the alignment was calculated based on the structural superposition and visualized using the CLC Main Workbench 7.5.1. (Qiagen Bioinformatics). The conservation below 30% identity is not colored, but a progressively darker red color represents conservation in the 30–100% range. The position of Arg-200 residue is shown with green boxes. The fractional conservation is represented below the alignment using bars and sequence conservation with sequence logo. G, superposition of the inhibitory motif residues Ser-31/Glu-35 in CdFic (dark yellow) with the CdFicSE/AA-ATP structure (gray). The ATP from the CdFicSE/AA-ATP structure is colored in gray, and a water molecule from the CdFicSE/AA-ATP structure is shown in red color interacting with both the γ-phosphate and the Fic motif Arg-174 and is in an overlapping position with the Glu-35 side chain of the CdFic structure.
dimer (Fig. 3A). The interface is formed by a short loop situated between helices $\alpha1$ and $\alpha'1$ (residues 51–59) in the N-terminal extension to the Fic core (Fig. 1B) and buries only ~6% (642 Å$^2$) of the total surface area (10,755 Å$^2$). However, as we have shown previously by size exclusion chromatography (SEC), the dimer is very stable and elutes as a single monodisperse peak (29).

There are six direct hydrogen bond interactions involving only backbone amide nitrogens and carbonyls in the loop, and three water molecules mediate contact between the two monomers (Fig. 3A). The loop conformation is structured mainly by residues Arg-56, His-57, and Asp-61, which tether the loop backbone through hydrogen bond interactions with Ser-37 and Thr-38 in the $\alpha_{nh}-\alpha'1$ loop. The conformation, but not the sequence, of this minimal dimerization interface is similar to the homologous BtFic (Protein Data Bank code 3CUC) and the human HYPE (56), both likewise situated on a small loop bridging helices $\alpha1-\alpha'1$ (Fig. 1C). The $\alpha1-\alpha'1$ loop in BtFic is also stabilized and tethered by hydrogen bonds to residues in the $\alpha_{nh}-\alpha'1$ loop but also shows no sequence conservation to the structurally equivalent residues in CdFic (Fig. 3A). Interestingly, although CdFic and BtFic share a highly similar relative orientation of the two monomers in the dimer, one monomer of the HYPE dimer is rotated by ~50° relative to the corresponding CdFic monomer as calculated by DynDom3D (57) (Fig. 3B). This rotation could either be a consequence of the HYPE crystal contacts or a result of the additional dimerization interface formed between residues in the $\alpha2$ helix of the two HYPE monomers (56).

To corroborate our previous SEC results, we probed the oligomeric state of CdFic in solution by SAXS on purified CdFic$^{SE/AA}$ (Fig. 4A). The pairwise distance distribution function $P(r)$ strongly suggests that CdFic is a dimer in solution as the longest particle dimension ($D_{max}$) is 106.4 Å and the longest dimension of the monomer in the crystals is ~50 Å. The CdFic$^{SE/AA}$ scattering profile was fitted and compared with the theoretical scattering profile of either the CdFic$^{SE/AA}$ dimer or monomer using CRYSOL (42) (Fig. 4A). The visual inspection shows considerably better fit to the dimer than the monomer, which is also supported by the calculated $\chi^2$ values of $\chi^2 = 3.20$ and $\chi^2 = 13.07$, respectively. However, the fit includes significant deviations that are likely explained by the presence of the 24-residue flap in each of the monomer units. These two loops of the dimer contribute to scattering of x-rays in solution but are flexible and not visible in the CdFic/CdFic$^{SE/AA}$ crystal structures without ATP (Fig. 1, A and D). These deviations were not resolved by fitting the CdFic$^{SE/AA}$-ATP dimer containing a well defined and closed flap ($\chi^2 = 3.92$), suggesting that the flap does not adopt a closed conformation in solution (Fig. 4B).

To determine the solution conformation of the flexible flap, we used recent developments that allow for rigid body modeling of known crystal structures to be combined with shape reconstruction of missing loops (58). A loop model was initially computed using Coral (58), resulting in an improved fit ($\chi^2 = 1.78$) but still containing significant deviations (data not shown). Subsequently, we used EOM, which enables analysis of flexible protein structures (46, 47). A pool of 10,000 independent models based on the flap sequence was initially generated, and a genetic algorithm was performed comparing the averaged theoretical scattering intensity from an ensemble of conformations with the experimental data. Two models were obtained by the ensemble, and CRYSOL (42) was used to compute a fit to the experimental SAXS data. Visual inspection reveals that the inclusion of the disordered flap modeled by EOM considerably improves the fit over the entire scattering angle range below 0.3 Å$^{-1}$ ($\chi^2 = 1.13$) (Fig. 4B).

Next, we performed three-dimensional ab initio shape reconstructions of the CdFic$^{SE/AA}$ dimer using DAMMIF (43). As models generated with P1 symmetry strongly suggest the presence of an extended two-domain particle, a P2 symmetry constraint was introduced. The mean normalized spatial discrep-
Crystal Structure of a Fic Protein from C. difficile

FIGURE 4. SAXS analysis. A, CdFic<sup>SE/AA</sup> SAXS scattering curve is shown in gray as relative log(intensity) versus inverse scattering angle. The Guinier plot (log I(q) versus q<sup>2</sup>) and a table with general SAXS parameters are both shown as insets. MW (monomer) is the mass calculated from the sequence, whereas MW (SAXS) is the mass estimated using the Porod volume. CRYSOl (42) fits between experimental SAXS data and the theoretical/calculated scattering profile as shown in colored curves. The fit to the CdFic<sup>SE/AA</sup> monomer is shown as a blue curve, whereas the fit to the CdFic<sup>SE/AA</sup> dimer is shown as a green curve. The resulting χ values (χ<sup>2</sup>) are shown next to the schematic models. B, the same experimental CdFic<sup>SE/AA</sup> SAXS scattering curve as in A is shown in gray color. The CRYSOl fit to the theoretical/calculated scattering profile of the CdFic<sup>SE/AA</sup>-ATP dimer structure including an ordered/closed flap is shown as a black curve. The CRYSOl fit to the CdFic dimer structure including the EOM (46, 47)-generated model of the flap is shown as a red curve. C, the three-dimensional shape reconstruction of the "ab initio" envelope calculated by DAPPmif using P2 symmetry is shown in transparent color. The CdFic<sup>SE/AA</sup> dimer structure with the two monomers shown in green and blue, respectively, is docked in the SAXS envelope. The EOM-generated reconstructions of the flexible flap are colored in red. D, the CdFic<sup>SE/AA</sup>-ATP crystal structure representing the closed/ordered conformation (red solid line) is aligned against the EOM-computed flap representing the open/flexible conformation (red spheres) with only one of the two EOM models shown for clarity. Superpositions of the BtFic (B. thetaotaomicron) and the HYpE (H. sapiens) flaps are shown in cyan and yellow, respectively.

ancy was 0.861, and the model with the lowest discrepancy (normalized spatial discrepancy, 0.734) was used as a representative (χ<sup>2</sup> = 1.008). Docking of the CdFic<sup>SE/AA</sup> crystal structure lacking the flap into the low resolution "ab initio" envelope shows that the envelope contains a larger volume and that this additional volume is well occupied by the EOM flap reconstructions representing the open flap conformation (Fig. 4C). Superposition of the solution conformation of the flap as determined by SAXS EOM analysis and the ATP-bound CdFic<sup>SE/AA</sup> crystal structure reveals that the flap adopts an open/flexible conformation in the free state and undergoes considerable transition of ~27 Å into a closed/ordered conformation upon ATP binding (Fig. 4D).

Cofactor Binding—To characterize ligand binding and specificity of CdFic and CdFic<sup>SE/AA</sup>, we used DSF (49, 59). By incubating 2 μM CdFic with ATP concentrations ranging between 0 and 17 mM, the melting temperature (T<sub>m</sub>) showed a maximum increase of stability (ΔT<sub>m</sub>) of 3.9 °C (Fig. 5A). The T<sub>m</sub> values determined at each ATP concentration were plotted versus the ATP concentration, resulting in a sigmoidal curve with a midpoint value around 2 mM and reaching a plateau at ~10 mM ATP (Fig. 5B). We also performed identical assays using CdFic with increasing concentrations of ADP, PP<sub>i</sub>, GTP, CTP, or UTP (Fig. 5, B and C). The largest increase in stability is obtained with ATP, ADP, and PP<sub>i</sub>, followed by GTP, which triggers an intermediate stability shift, whereas CTP/UTP pyrimidines do not significantly induce CdFic stability. Hence, the PP<sub>i</sub>, moiety, which alone is able to induce stability significantly, is not sufficient for pyrimidine nucleotides to stabilize CdFic. Finally, to assess the importance of the inhibitory motif residues Ser-31 and Glu<sub>inh</sub>-35 on ligand binding, we performed identical assays using the CdFic<sup>SE/AA</sup> mutant (Fig. 5, D and E). Interestingly, the CdFic<sup>SE/AA</sup> nucleotide binding profile is almost identical to that of CdFic (Fig. 5F).

CdFic Autoadenylation—In the absence of a known physiological protein target, autoadenylation is a useful read-out to assess adenylation activity of Fic domain proteins (28). To determine whether CdFic is able to not only bind ATP but also catalyze AMP transfer, we incubated the purified protein with radioactively labeled [α-32P]ATP before visualizing labeled gel bands by phosphorimaging. We found that, despite the presence of an intact proposed inhibitory motif, CdFic is able to catalyze autoadenylation in vitro (Fig. 6A). We also tested for self-labeling using the CdFic<sup>SE/AA</sup> inhibitory mutant and found that the activity is essentially identical to that of the wild type protein and therefore independent of the inhibitory motif in...
In accordance with the DSF assay and the CdFicSE/AA-ATP crystal structure. As a negative control, replacement of the highly conserved catalytic Fic motif His-163 with an alanine shows no sign of autoadenylation activity, confirming that activity is driven by the Fic motif. Also, to determine whether dimerization is important for the activity, we replaced His-57 involved in stabilizing the dimerization interface (Fig. 3A) with alanine, expecting that this would disrupt dimerization. However, SEC revealed that H57A elutes as a single peak corresponding to a dimer as described previously for CdFic and CdFicSE/AA (29). Surprisingly, although dimerization of this mutant is not disrupted, we found that the H57A point mutation enhances self-labeling dramatically (Fig. 6A). This result further validates the non-obstructed ATP conformation seen in the CdFicSE/AA-ATP crystal structure. However, although autoadenylation activity of CdFic is independent of the αinh helix, the activity is still less than that for both NmFic and the dramatically more active αinh mutant NmFicE186G when assayed under identical conditions (Fig. 6D).

To identify the autoadenylation acceptor site, we performed mass spectrometry analysis of CdFicSE/AA incubated with ATP. The location of the acceptor site was traced to a single tryptic peptide (supplemental Fig. 1, A–C). The MS2 spectra narrowed down the site to either Ser-37 or Thr-38, both residues located in the αinh helix, which resulted in the identification of the same, but unmodified, peptide. An additional control experiment was performed by incubation of an isotope-labeled ATP, also identifying Thr-38 as the acceptor site based on the MS2 spectra (supplemental Fig. 1, D–F). Unfortunately, in both cases, most of the obtained MS2 spectra lacked information about the y13-y14 and b4-b5 ions, which made it difficult to determine the exact location of the AMP group. To determine the exact acceptor site, we substituted either residue Ser-37 or Thr-38 with alanine before assaying the mutants for autoadenylation activity (Fig. 6E). Although CdFicS37A catalyzes self-labeling comparable with wild type protein, CdFicT38A is inactive, thus identifying Thr-38 as the AMP acceptor site. Two acceptor sites were previously mapped for NmFic with the primary acceptor site being Tyr-183 inside the inhibitory motif and an additional acceptor site, Tyr-188, located to the αinh-α1 loop (21).
Tyr-183 primary acceptor site in NmFic (Fig. 6F). The lack of a hydroxyl acceptor at this position may thus explain why this is not a CdFic acceptor site. In contrast, the hydroxyl group of the Tyr-188 secondary acceptor residue in NmFic is structurally in the same position as the hydroxyl group of the Thr-38 acceptor residue in CdFic (Fig. 6, F and G). As the Thr-38 side chain hydroxyl group of CdFic is positioned 15.5 Å away from the ATP α-phosphate and is solvent-exposed, autoadenylation likely occurs intermolecularly and only after partial rearrangement of the αinh-α1 loop (Fig. 6H). Interestingly, the Thr-38 side chain forms hydrogen bonds to the side chains of Asp-61 and the dimerization loop residues Arg-56 and His-57. Hence, the disruption of the hydrogen bond between Thr-38 and His-57 in the H57A mutant could result in Thr-38 becoming more solvent-accessible, thereby explaining why this mutant shows a dramatic increase in autoadenylation activity (Fig. 6A). Although placed near the dimerization interface, the T38A mutant, like H57A, does not disrupt dimer formation.

Finally, Thr-38 is conserved in both HYPE and BtFic (Fig. 6E), which suggests that these two Fic proteins could have autoadenylation targets similar to CdFic. However, it was recently shown that autoadenylation of the human Fic protein HYPE occurs outside of the Fic core domain (16). Hence, CdFic is able to catalyze AMP transfer in vitro, and the autoadenylation activity, in agreement with the CdFicSE/AA-ATP structure, is independent of the inhibitory motif.

Discussion

The results presented here characterize a novel adenylylating class II Fic domain protein from the important human pathogen C. difficile. This is the first Fic domain protein identified with autoadenylation activity incompatible with the proposed...
model of inhibition for class I–III Fic domain proteins (21). Although structurally similar to other Fic proteins and exhibiting the presence of α\textsubscript{inh}, we show that wild type CdFic harbors \textit{in vitro} autoadenylylation activity, which is not enhanced by introduction of point mutations in the inhibitory motif (Fig. 6A). This is supported by the combined crystal structures of CdFic and CdFic\textsuperscript{SE/A}ATP, which reveal that ATP is bound in a conformation that is not obstructed by the inhibitory helix (Fig. 1). The structures of CdFic also provide evidence for a dimer formation and for the movements of the CdFic flap upon ATP binding. We have calculated the low resolution SAXS envelope of the dimer as well as shape reconstructions of the flap, representing the open/disordered conformation of the flap in solution. Superposition against the closed/ordered flap in the crystal structure of CdFic\textsuperscript{SE/A}-ATP complex highlights the large active site closure that occurs upon ATP binding (Fig. 4D). Moreover, after prolonged incubation with ATP, SEC provides no indication of monomer formation of CdFic, suggesting that autoadenylylation does not disrupt the dimer (results not shown). In the active site, the base and the ribose adopt a canonical position, whereas the phosphates adopt a different path where γ-phosphate coordination is not overlapping with the side chain of Ghu\textsubscript{inh} in the inhibitory motif (Fig. 2, C and G).

Notably, the α- and β-phosphates of the ATP are situated on the opposite side of the active site cleft compared with those seen in other Fic domains. This placement of the ATP phosphates is likely explained by the salt bridge formed between the α-phosphate and Arg-200 (Fig. 2E). No other structurally characterized Fic domains are shown to have a similarly positioned arginine in the Fic core on the opposite side of the active site relative to the conserved Fic motif (Fig. 2F). In other structures published to date, the α-phosphate instead forms a salt bridge to the conserved asparagine in the GNG anion hole (Fig. 2D).

Also, the Mg\textsuperscript{2+} ion in other Fic-ATP-Mg\textsuperscript{2+} structures is coordinated between the α- and the β-phosphates. However, in the CdFic\textsuperscript{SE/A}-ATP structure, the Mg\textsuperscript{2+} ion is instead coordinated by the β- and the γ-phosphates (Fig. 2C). This type of Mg\textsuperscript{2+} coordination seen in CdFic is in line with the suggested adenylylation reaction mechanism based on the crystal structure of the BepA-Mg\textsuperscript{2+}-PP\textsubscript{i} complex where Mg\textsuperscript{2+}-PP\textsubscript{i} appears to be a part of the product (23, 60). Conversely, this particular Mg\textsuperscript{2+} coordination is also seen in other ATP-binding enzymes where Mg\textsuperscript{2+} chelates the γ- and β-phosphate groups to lower the threshold either for γ-phosphoryl transfer to a nucleophilic (-OH) group or for hydrolyzing ATP to ADP to release energy to drive a subsequent reaction (61).

We also attempted to soak wild type CdFic crystals with ATP, but because of limited diffraction quality of the crystals we did not succeed to obtain a crystal structure of this complex. However, the biological significance of the CdFic\textsuperscript{SE/A}-ATP structure is supported by the following observations. (i) A Mg\textsuperscript{2+} ion is tightly coordinated in the active site. (ii) Replacement of Arg-200 with an alanine significantly reduces autoadenylylation activity, demonstrating the catalytic importance of this residue. (iii) The ATP molecule is not engaged in any crystal packing interactions. (iv) The same ATP conformation was seen in a 3.1-Å structure of the CdFic\textsuperscript{SE/A}-ATP complex (results not shown), which was solved independently of the presented 2.5-Å complex structure.

In further support of the inhibitory motif-independent mechanism of CdFic, we observe no differences in DSF binding assays of ATP to wild type CdFic and the CdFic\textsuperscript{SE/A} mutant (Fig. 5). Recently, DSF was demonstrated as an important method for studying inhibitory motif dependence where the human class II Fic domain protein HYPE and its Glu\textsubscript{inh} mutant exhibited significant differences in thermal shifts (56). HYPE is known to autoadenylate as well as adenylate the chaperone BiP, and both activities were shown to be highly dependent on the inhibitory motif (16). Our CdFic DSF results are considerably different from those of HYPE because only ADP, but none of the triphosphate nucleotides or PP\textsubscript{i} increases the stability of HYPE. In contrast, the Glu\textsubscript{inh} mutant of HYPE behaves similarly to both CdFic and CdFic\textsuperscript{SE/A} as ATP, ADP, and PP\textsubscript{i} produce the largest thermal shifts, whereas the pyrimidine nucleotides produce the smallest. We also tested other nucleotides in the same assay and found that ATP and ADP induce the highest thermal shifts, suggesting binding specificity for the adenosine base (Fig. 5). In further support of this, we conducted autoadenylylation assays using radioactively labeled CTP and observed no self-labeling (Fig. 6D). These results combined suggest that CdFic is an adenylase factor \textit{in vivo} with a nucleotide binding profile of CdFic independent of the inhibitory motif. The structural basis for the non-obstructed autoadenylylation reaction mechanism of CdFic is summarized schematically in Fig. 7.

It is currently unknown what mechanisms and factors relieve the autoinhibition of class I–III Fic proteins. In addition, the autoinhibition observed in most studies was incomplete as Fic domains in general harbor activity \textit{in vitro} although typically much less compared with the point mutants of the inhibitory motif (Fig. 6D) (16, 28). Although the autoadenylylation activity for both CdFic and CdFic\textsuperscript{SE/A} is low compared with NmFic\textsuperscript{E186C} (Fig. 6D), we observed a dramatic increase in activity upon the H57A substitution positioned in the dimerization interface (Fig. 6A). This could be due to a disruption of the hydrogen bond between the side chains of residues His-57 and Thr-38, thereby making the acceptor site more easily accessible for autoadenylylation (Fig. 6G). Alternatively, although SEC reveals that H57A still forms a stable dimer in solution, the substitution may affect the conformation of the dimer. The importance of dimerization for autoadenylylation activity was recently demonstrated for human HYPE, which showed a dramatic decrease in activity when residues inside or near the dimerization loop were substituted (56). Nevertheless, the increase in activity caused by the H57A mutant suggests that an activation mechanism that needs to take place before CdFic can carry out its function might exist and that this activation mechanism is different from the autoinhibition by α\textsubscript{inh}.

The physiological protein target of CdFic remains unknown. Attempts to identify the target by incubating purified recombinant CdFic, CdFic\textsuperscript{SE/A}, as well as the more active CdFic\textsuperscript{H57A} together with [α-32P]ATP and lysates of either human Caco-2 or \textit{C. difficile} cells or with a broad selection of purified RhoGTPases have so far been unsuccessful. Possibly, the protein target has to be found elsewhere, or an alternative activation
Crystal Structure of a Fic Protein from C. difficile

FIGURE 7. Non-obstructed ligand binding. Schematic models for inhibitory motif obstructed (left) and non-obstructed (right) autoadenylation mechanism are shown. In previously described Fic proteins, the inhibitory helix αinh and/or the associated inhibitory residue Gluinh must move away from the obstructive position to accommodate a salt bridge between the terminal phosphate of ATP and the C-terminal arginine of the Fic motif. However, in the non-obstructed autoadenylation mechanism for CdFic, the ATP is pulled toward the opposite side of the active site cleft. Consequently, the position of Gluinh is not obstructing proper positioning of the ATP phosphates, and the autoadenylation activity is not inhibited. The rearrangement of the ATP path alters the position of the phosphates relative to the Mg$^{2+}$ ion. The Mg$^{2+}$ is shown in red, ATP is in black, the anion hole is in blue, and the inhibitory helix is in dark yellow. Highly conserved residues involved in ATP binding are indicated. For CdFic, Arg-200 that specifically interacts with the α-phosphate is also shown.

mechanism that needs to take place before CdFic can carry out its function might exist. Despite showing that ATP binding and autoadenylation by CdFic are independent of the inhibitory motif, we cannot rule out the possibility that the αinh of CdFic might still have a function in inhibiting the adenylolation activity against the currently unknown target. However, this would be unusual because the εinh is known to regulate both autoadenylation and adenylolation in other Fic proteins with known protein targets, such as HYPE (16) and NmFic (62).

In addition, we are unable to completely rule out that CdFic is a kinase or modifies a target protein with an ADP moiety. However, we have looked for possible kinase activity of CdFic in lysed Caco-2 cells with no positive outcome. Furthermore, using mass spectrometry, we detected an AMP moiety attached to Thr-38 of CdFic upon incubation with ATP (Fig. 6E and supplemental Fig. 1), providing important evidence that the scissile bond is situated between the α- and β-phosphates. The AMP acceptor site structurally aligns to one of the two AMP acceptor sites of the inhibitory motif-dependent NmFic (Fig. 6, F and G). This strongly suggests that CdFic catalyzes adenylolation or AMPylation and that the unusual behavior of CdFic in relation to the inhibitory motif is not due to differences in the position of the acceptor site.

Currently, fewer than 10 other unique Fic domain structures with ATP in the binding site are deposited in the Protein Data Bank. Despite this very limited structural knowledge and the high conservation of the Fic motif, Fic domains have been shown to have high diversity. For instance, the Legionella effector AnkX (25) utilizes CDP-choline to catalyze phosphocholine modifications, whereas IbpA has affinity for both ATP and GTP (24). These studies show that the Fic core, which contains weak sequence conservation, is able to confer additional specificity to a structurally conserved Fic motif evolved to coordinate the ribose and the phosphates of nucleotides. In line with this and because the conserved Fic motif in CdFic is structurally similar to other known Fic enzymes, the Fic core of CdFic likely alters the ATP binding of the Fic motif to overcome the obstruction by the inhibitory motif. These new insights into the first characterized Fic domain protein from a Gram-positive bacterium clearly illustrate the diversity of this very large family despite having the same structural fold. They also provide important steps toward revealing the mechanistic function, the protein target, and hence the physiological role of CdFic as well as the potential framework to identify possible other inhibitory motif-independent Fic domain proteins.

Author Contributions—E. D., H. C. v. L., and R. J. conceived and designed the experiments. E. D. performed the SAXS experiments. H. A. designed and performed the DSF experiments. E. D., D. H. W., and R. J. performed the crystallization experiments, x-ray data collection, and structure solution. O. Ø. performed the mass spectrometry analyses. H. C. v. L., J. C., O. I. K., and P. J. H. performed the cloning and site-directed mutagenesis as well as the in vitro adenylation assays. E. D., H. C. v. L., H. A., and R. J. analyzed the data. E. D. and R. J. wrote the paper. All authors approved the final version of the manuscript.

Acknowledgments—We thank Wip Klaas Smit and Sjaak van Voorden (Leiden University Medical Center, Netherlands) for help with cloning. We thank the group of Tilman Schirmer at Biozentrum, University of Basel, for sending us the NmFic and NmFicSE/AA clones. We also acknowledge Giancarlo Tria (Nanyang Technological University, Singapore) for advice regarding EOM SAXS analysis, Seema Mattoo for helpful discussions and carefully reading the manuscript, Tomás Plivelic at SAXS beamline I911-4 MAX-II Laboratory for help with SAXS data collection, the MX beamline staff at I911-2 at MAX-II Laboratory, and the MX beamline staff at ID23-2 and ID29 at European Synchrotron Radiation Facility (Grenoble, France) for help with x-ray diffraction data collection.

References
1. Poutanen, S. M., and Simor, A. E. (2004) Clostridium difficile-associated diarrhea in adults. CMAJ 171, 51–58
2. Elliott, B., Chang, B. J., Golledge, C. L., and Riley, T. V. (2007) Clostridium difficile-associated diarrhea. Int. Med. J. 37, 561–568
3. McFarland, L. V., Beneda, H. W., Claridge, J. E., and Raugi, G. J. (2007) Implications of the changing face of Clostridium difficile disease for health care practitioners. Am. J. Infect. Control 35, 237–253
4. Just, I., Selzer, J., von Eichel-Streiber, C., and Aktories, K. (1995) The low molecular mass GTP-binding protein Rho is affected by toxin A from...
Crystal Structure of a Fic Protein from C. difficile

C. difficile. J. Clin. Investig. 95, 1026–1031
5. Just, I., Wilm, M., Selzer, J., Rex, G., von Eichel-Streiber, C., Mann, M., and Aktories, K. (1995) The enterotoxin from C. difficile (ToxA) monoglucosylates the Rho proteins. J. Biol. Chem. 270, 13932–13936
6. Lyras, D., O’Connor, I. R., Howarth, P. M., Sambol, S. P., Carter, G. P., Phumoomna, T., Poon, R., Adams, V., Vedantam, G., Johnson, S., Gerdin, D. N., and Rood, J. I. (2009) Toxin B is essential for virulence of C. difficile. Nature 458, 1176–1179
7. Kuehne, S. A., Cartman, S. T., Heap, J. T., Kelly, M. L., Cockayne, A., and Minton, N. P. (2010) The role of toxin A and toxin B in C. difficile infection. Nature 467, 711–713
8. Lyerly, D. M., Saum, K. E., MacDonald, D. K., and Wilkins, T. D. (1985) Effects of C. difficile toxins given intragastrically to animals. Infect. Immun. 47, 349–352
9. Quesada-Gómez, C., López-Ureña, D., Acuña-Amador, L., Villalobos-Zúñiga, M., Du, T., Freire, R., Guzmán-Verri, C., del Mar Gamoa-Corona, N., Lawley, T. D., Moreno, E., Mulvey, M. R., de Castro Brito, G. A., Rodríguez-Cavallini, E., Rodríguez, C., and Chaves-Olarte, E. (2015) Emergence of an outbreak-associated C. difficile difficile variant with increased virulence. J. Clin. Microbiol. 53, 1216–1226
10. Yearborg, M. L. L., Yli, K., Kinch, L. N., Grishin, N. V., Ball, H. L., and orth, K. (2009) AMPylation of Rhb GTPases by Vibrio VopS disrupts effector binding and downstream signaling. Science 323, 269–272
11. Worby, C. A., Mattoo, S., Kruger, R. P., Corbel, L. B., Koller, A., Mendez, J. C., Zeikias, B., Lazar, C., and Dixon, J. E. (2009) The fic domain: regulation of cell signaling by adenyllylation. Mol. Cell 34, 93–103
12. Garcia-Pino, A., Zenkin, N., and Loris, R. (2014) The many faces of Fic: structural and functional aspects of Fic enzymes. Trends Biochem. Sci. 39, 121–129
13. Müller, M. P., Peters, H., Bluemer, J., Blankenfeldt, W., Goody, R. S., and Garcia-Pino, A., Zenkin, N., and Loris, R. (2014) The many faces of Fic: structural and functional aspects of Fic enzymes. Trends Biochem. Sci. 39, 121–129
14. Walter, M. P., Peters, H., Bluemer, J., Blankenfeldt, W., Goody, R. S., and Itzen, A. (2010) The Legionella effector protein DrsA AMPylates the membrane traffic regulator Rab1. Science 329, 946–949
15. Rahman, M., Ham, H., Liu, X., Sugura, Y., orth, K., and Krämer, H. (2012) Visual neurotransmission in Drosophila requires expression of Fic in glial capitate projections. Nat. Neurosci. 15, 871–875
16. Ham, H., Woolery, A. R., Tracy, C., Stenesci, D., Krämer, H., and orth, K. (2014) Unfolded protein response-regulated dFic reversibly AMPylates BIP during endoplasmic reticulum homeostasis. J. Biol. Chem. 289, 36059–36069
17. Sanyal, A., Chen, A. J., Nakayasu, E. S., Lazar, C. S., Zbornik, E. A., Worby, C. A., Koller, A., and Mattoo, S. (2015) A novel link between Fic (filamentation induced by cAMP)-mediated adenylylation/AMPylation and the inhibitory mechanism and competent ATP binding mode for adenylyltransferases with Fic fold. PLoS One 8, e64901
18. Welner, D., Dedic, E., van Leeuwen, H. C., Kuijper, E., Bjerrum, M. J., Emsley, P., Lohkamp, B., Scott, W. G., and Cowtan, K. (2010) Features and development of Coot. Acta Crystallogr. D Biol. Crystallogr. 66, 486–501
19. Adams, P. D., Afonine, P. V., Bunkóczi, G., Chen, V. B., Davis, I. W., Echols, N., Headd, J. J., Hung, L. W., Kapral, G. J., Grosjean-Kurk, R. W., McCoy, A. J., Moriarty, N. W., Oeffner, R., Read, R. J., Richardson, D. C., Richardson, J. S., Terwilliger, T. C., and Zwart, P. H. (2010) PHENIX: a comprehensive Python-based system for macromolecular structure solution. Acta Crystallogr. D Biol. Crystallogr. 66, 213–221
20. Painter, J., and Merritt, E. A. (2006) Optimal description of a protein structure in terms of multiple groups undergoing TLS motion. Acta Crystallogr. D Biol. Crystallogr. 62, 439–450
21. Delano, W. L. (2002) The PyMOL Molecular Graphics System, Schrödinger, LLC, New York
22. Krissinel, E., and Henrick, K. (2007) Inference of macromolecular assemblies from crystalline state. J. Mol. Biol. 372, 774–797
23. Konarev, P. V., Volkov, V. V., Sokolova, A. V., Koch, M. H. J., and Svergun, D. I. (2003) PRIMUS: a Windows PC-based system for small-angle scattering data analysis. J. Appl. Crystallogr. 36, 1277–1282
24. Svergun, D., Barbarato, C., and Koch, M. H. J. (1995) CRYOSOL—a program to evaluate x-ray solution scattering of biological macromolecules from atomic coordinates. J. Appl. Crystallogr. 28, 768–773
25. Franke, D., and Svergun, D. I. (2009) DAMMIF, a program for rapid ab-initio shape determination in small-angle scattering. J. Appl. Crystallogr. 42, 342–346
26. Volkov, V. V., and Svergun, D. I. (2003) Uniqueness of ab initio shape determination in small-angle scattering. J. Appl. Crystallogr. 36, 860–864
27. Wrighers, W. (2010) Using Situs for the integration of multi-resolution structures. Biophys. Rev. 2, 21–27
28. Bernardó, P., Mylonas, E., Petoukhov, M. V., Blackledge, M., and Svergun, D. I. (2007) Structural characterization of flexible proteins using small-angle x-ray scattering. J. Am. Chem. Soc. 129, 5656–5664
29. Tria, G., Mertens, H. D., Kachala, M., and Svergun, D. I. (2015) Advanced ensemble modelling of flexible macromolecules using x-ray solution scatter-
Crystal Structure of a Fic Protein from C. difficile

48. Pettersen, E. F., Goddard, T. D., Huang, C. C., Couch, G. S., Greenblatt, D. M., Meng, E. C., and Ferrin, T. E. (2004) UCSF Chimera—a visualization system for exploratory research and analysis. J. Comput. Chem. 25, 1605–1612

49. Niesen, F. H., Berglund, H., and Vedadi, M. (2007) The use of differential scanning fluorimetry to detect ligand interactions that promote protein stability. Nat. Protoc. 2, 2212–2221

50. Shevchenko, A., Tomas, H., Havlis, J., Olsen, J. V., and Mann, M. (2006) In-gel digestion for mass spectrometric characterization of proteins and proteomes. Nat. Protoc. 1, 2856–2860

51. Rappsilber, J., Ishihama, Y., and Mann, M. (2003) Stop and go extraction tips for matrix-assisted laser desorption/ionization, nanoelectrospray, and LC/MS sample pretreatment in proteomics. Anal. Chem. 75, 663–670

52. Cox, J., and Mann, M. (2008) MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. Nat. Biotechnol. 26, 1367–1372

53. Cox, J., Neuhauer, N., Michalski, A., Scheltema, R. A., Olsen, J. V., and Mann, M. (2011) Andromeda: a peptide search engine integrated into the MaxQuant environment. J. Proteome Res. 10, 1794–1805

54. Li, Y., Al-Eryani, R., Yarbrough, M. L., Orth, K., and Ball, H. L. (2011) Characterization of AMPylation on threonine, serine, and tyrosine using mass spectrometry. J. Am. Soc. Mass Spectrom. 22, 752–761

55. Morris, A. L., MacArthur, M. W., Hutchinson, E. G., and Thornton, J. M. (1992) Stereochemical quality of protein structure coordinates. Proteins 12, 345–364

56. Bunney, T. D., Cole, A. R., Broncel, M., Esposito, D., Tate, E. W., and Katan, M. (2014) Crystal structure of the human, FIC-domain containing protein HYPE and implications for its functions. Structure 22, 1831–1843

57. Poornam, G. P., Matsumoto, A., Ishida, H., and Hayward, S. (2009) A method for the analysis of domain movements in large biomolecular complexes. Proteins 76, 201–212

58. Petoukhov, M. V., Franke, D., Shkumatov, A. V., Tria, G., Kikhney, A. G., Gajda, M., Gorba, C., Mertens, H. D., Konarev, P. V., and Svergun, D. I. (2012) New developments in the ATSAS program package for small-angle scattering data analysis. J. Appl. Crystallogr. 45, 342–350

59. Boivin, S., Kozak, S., and Mejiers, R. (2013) Optimization of protein purification and characterization using Thermofluor screens. Protein Expr. Purif. 91, 192–206

60. Palanivelu, D. V., Goepfert, A., Meury, M., Guye, P., Dehio, C., and Schirmer, T. (2011) Fic domain-catalyzed adenyllylation: insight provided by the structural analysis of the type IV secretion system effector BepA. Protein Sci. 20, 492–499

61. Matte, A., Tari, L. W., and Delbaere, L. T. (1998) How do kinases transfer phosphoryl groups? Structure 6, 413–419

62. Stanger, F. V., Burmann, B. M., Harms, A., Aragão, H., Mazur, A., Sharpe, T., Dehio, C., Hiller, S., and Schirmer, T. (2016) Intrinsic regulation of FIC-domain AMP-transferases by oligomerization and automodification. Proc. Natl. Acad. Sci. U.S.A. 113, E529–E537

63. Wallace, A. C., Laskowski, R. A., and Thornton, J. M. (1995) LIGPLOT: a program to generate schematic diagrams of protein-ligand interactions. Protein Eng. 8, 127–134

64. Gille, C., and Frömml, C. (2001) STRAP: editor for STRuctural Alignments of Proteins. Bioinformatics 17, 377–378

65. Karplus, P. A., and Diederichs, K. (2012) Linking crystallographic model and data quality. Science 336, 1030–1033

66. Diederichs, K., and Karplus, P. A. (1997) Improved R-factors for diffraction data analysis in macromolecular crystallography. Nat. Struct. Biol. 4, 269–275