Fic domain-catalyzed adenylylation: Insight provided by the structural analysis of the type IV secretion system effector BepA

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Received 25 October 2010; Revised 2 December 2010; Accepted 3 December 2010

DOI: 10.1002/pro.581

Published online 6 January 2011 proteinscience.org

Abstract: Numerous bacterial pathogens subvert cellular functions of eukaryotic host cells by the injection of effector proteins via dedicated secretion systems. The type IV secretion system (T4SS) effector protein BepA from *Bartonella henselae* is composed of an N-terminal Fic domain and a C-terminal *Bartonella* intracellular delivery domain, the latter being responsible for T4SS-mediated translocation into host cells. A proteolysis resistant fragment (residues 10–302) that includes the Fic domain shows autoadenylylation activity and adenylyl transfer onto Hela cell extract proteins as demonstrated by autoradiography on incubation with α -[³²P]-ATP. Its crystal structure, determined to 2.9-Å resolution by the SeMet-SAD method, exhibits the canonical Fic fold including the HPFxxGNGRxxR signature motif with several elaborations in loop regions and an additional β -rich domain at the C-terminus. On crystal soaking with ATP/Mg²⁺, additional electron density indicated the presence of a PP_i/Mg²⁺ moiety, the side product of the adenylylation reaction, in the anion binding nest of the signature motif. On the basis of this information and that of the recent structure of IbpA(Fic2) in complex with the eukaryotic target protein Cdc42, we present a detailed model for the ternary complex of Fic with the two substrates, ATP/Mg²⁺ and target tyrosine. The model is consistent with an in-line nucleophilic attack of the deprotonated side-chain hydroxyl group onto the α -phosphorus of the nucleotide to accomplish AMP transfer. Furthermore, a general, sequence-independent mechanism of target positioning through antiparallel β -strand interactions between enzyme and target is suggested.

Keywords: FIC domain; AMPylation; adenylylation; AMP transfer; type IV secretion system

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Additional Supporting Information may be found in the online version of this article.

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Grant sponsor: Swiss National Science Foundation; Grant number: 3100a0-109925; Grant sponsor: Howard Hughes Medical Institute; Grant number: 55005501; Grant sponsor: SystemsX.ch Swiss Initiative for Systems Biology; Grant number: 51RT O_126008 (InfectX)

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Introduction

Adenylation, that is, AMP-transfer from an ATP substrate onto a target molecule, is a common process in biology. This enzymatic reaction occurs during nucleotide polymerization and on priming carboxylate substrates for further chemical modification as, for example, catalyzed by acyl-CoA synthetase.¹ However, proteins also can become post-translationally modified by attaching the AMP moiety onto various types of side-chains. In this way, a lysyl side-chain is transiently adenylylated in polynucleotide ligase, followed by adenylyl transfer onto the substrate.² Glutamine synthetase adenylyl transferase controls the activity of an important metabolic enzyme by tyrosyl modification.³ A related protein from the pathogen *Legionella*, DrrA, adenylylates the membrane traffic regulator Rab1b on translocation into eukaryotic host cells.⁴ Only recently, the Fic (filamentation induced by cAMP) domain (Pfam 24.0⁵) found in almost all kingdoms of life and annotated in currently more than 2000 sequences has been recognized to catalyze adenylyl transfer onto specific protein hydroxyl side-chains of interacting target proteins. This has been demonstrated for two bacterial effector proteins carrying a Fic domain, VopS of *Vibrio parahaemolyticus*, and IbpA of *Histophilus somni*, which on translocation into mammalian host cells inactivate small GTPases of the Rho-family by adenylation of conserved threonine or tyrosine residues, respectively, resulting in a collapse of the cellular actin cytoskeleton.^{6,7}

Fic-domain containing effector proteins are also encoded by *Bartonella henselae*.⁸ This pathogen translocates via a dedicated protein secretion system, the type IV secretion system VirB, seven effector proteins known as BepA-BepG into human endothelial cells.⁹ The resulting cellular changes include rearrangements of the actin cytoskeleton, the inhibition of programmed cell death (apoptosis), the activation of a proangiogenic process, and the initiation of an inflammatory response.¹⁰ All seven Beps show one or several *Bartonella* intracellular delivery (BID) domains in the C-terminal part that are responsible for protein delivery into host cells,⁹ whereas the N-terminal part of the homologous BepA, BepB, and BepC proteins comprise a Fic domain (see also Fig. 1, top). The BID domain of BepA mediates inhibition of apoptosis,¹¹ whereas the precise function of the N-terminal Fic domain is yet unknown. Here, we present the crystal structure of the BepA Fic domain in complex with pyrophosphate, the side product of the adenylation reaction. Combined with other published structural information,¹² the structure of a generic ternary substrate complex is inferred and a detailed catalytic mechanism of the adenylyl transfer onto the target hydroxyl group is proposed.

Figure 1. Crystal structure of BepA_{tr} (residues 12–302) and scheme of the BepA sequence (top). The following color code is used throughout: red, core of Fic domain as defined by Pfam⁵; blue, peptide segment (103–105) in extended conformation that has been shown to interact with target protein/peptide in structural homologs IbpA,¹² AvrB,¹⁹ and SO2466¹³; light-grey, part of the flap that is not resolved by electron density (106–111, light-grey); yellow, HPFxxGNG signature motif; gold, OB-fold (see also Supporting Information Fig. S2); magenta, remainder of structure. Secondary structure elements are numbered sequentially, α , α -helices, η , 3_{10} -helices, and β , β -strands.

Results and Discussion

Crystal structure of a BepA fragment comprising the Fic domain

An N-terminal His₆-tagged construct of full-length BepA (64 kDa) was expressed in *E. coli* and was found to be proteolytically truncated at the C-terminus to yield a fragment (BepA_{tr}) with a mass of about 40 kDa as evidenced by size-exclusion chromatography and anti-His Western blot. Removal of the His-tag, which proved essential for crystallization, was achieved by proteolytic treatment with thrombin. The two molecules of the asymmetric unit form a weak, probably nonphysiological, symmetric dimer (Supporting Information Fig. S1). Figure 1 shows the overall structure of BepA_{tr} with residues 12–302 well defined by electron density except for residues 106–111 of a loop close to the signature motif (flap) and a few side-chains. The structure can be grouped in an N-terminal, predominantly α -helical part (12–245), and the remaining C-terminal end exhibiting the OB (oligonucleotide/oligosaccharide binding)-fold (Supporting Information Fig. S2). Residues 62–177 [helices α 2– α 5, see also Fig. 2(A)] are classified as Fic domain in the Pfam database.⁵ There is a small

Figure 2. Structural comparison of (A) BepA_{tr} (PDB code: 2vy3) with a chloride ion bound to the N-cap of helix $\alpha 5$ and (B) lbpA(Fic2, H3717A) in complex with Cdc42 (green) that has its modified AMP-Y₃₂ residue bound to the Fic active site (PDB code: 3n3v¹²). Same color code as in Figure 1, view rotated by 90° about the vertical direction. Important residues are labeled. The OB-domain of BepA_{tr} has been removed for clarity.

β -sheet ($\beta 1/\beta 2$) inserted into the $\alpha 2$ – $\alpha 3$ loop that is not part of the Fic profile. The Fic core structure is flanked N-terminally by $\alpha 1$ and irregular loop structure. C-terminally it is followed by a three helix bundle ($\alpha 6$ – $\alpha 8$) and $\beta 3$ that is an extension of the β -sheet of the OB-fold domain.

Figure 2(A) shows that the H₁₅₉PFxxGNG signature motif of the Fic profile locates to the $\alpha 4$ – $\alpha 5$ loop and the following N-cap of helix $\alpha 5$. A chloride ion is bound to the N-cap in both chains of the asymmetric unit. The N₁₆₅ side-chain forms two H-bonds with the backbone amide and carbonyl group of the phenylalanine residue [F₁₆₁, see also Fig. 3(A)] and may thus be critical for defining the exact geometry of the loop. The phenylalanine side-chain is tucked away in the hydrophobic core of the domain.

Structural comparisons

Several structures of Fic proteins have been determined by structural genomics initiatives (PDB codes: 2f6s, 2g03, 3cuc, and 3eqx¹³). BepA_{tr} exhibit close structural resemblance with these proteins (Z-scores 11–13 as determined by Dali,¹⁴ see also Supporting Information Figs. S3 and S4) showing the same arrangement for helices $\alpha 1$ to $\alpha 7$. Helix $\alpha 8$ is packed peripherally, together with $\beta 3$, onto $\alpha 6$ and $\alpha 7$ and connects to the OB-domain (Fig. 1). In the Fic proteins and in BepA, an extended HPFxxGNGRxxR motif is strictly obeyed (Supporting Information Fig. S4a). More distant structural homologs are the type III effectors AvrB and VopS, and the Doc toxin (Supporting Information Figs. S3 and S4) that give moderate Z-scores of 8, 5, and 6, respectively. Interestingly, in

BepA_{tr} as in Doc toxin, a helix, that runs antiparallelly to $\alpha 5$ and $\alpha 1$ is missing. This leaves a groove in the structure that is taken up by the Phd antitoxin in the Doc/Phd complex.¹⁵ Notably, there is no overall significant sequence similarity of AvrB and Doc with BepA and the sequences of the signature loop are degenerated (Supporting Information Fig. S4). Still these segments show the canonical conformation (Supporting Information Fig. S3). A thorough structural comparison of all these structural homologs of BepA has appeared recently.¹⁶ The recently reported structure of the Fic2 domain of lbpA¹² [Fig. 2(B)] also exhibits significant (Z = 8) similarity with BepA and the other Fic proteins (Supporting Information Fig. S4B).

BepA_{tr}/PP_i/Mg²⁺ product complex

At the time the BepA_{tr} structure was solved, there was no clue about the molecular function of Fic domains. However, the N-cap of $\alpha 5$ (residues 165–167) together with the preceding residue G₁₆₄ appeared well suited for polyanion binding. In fact, the three backbone amide nitrogens of residues G₁₆₄, N₁₆₅, and G₁₆₆ form a well-defined so-called anion binding “nest” of type LR¹⁷ as noted before for the Doc protein.¹⁵ Therefore, BepA_{tr} crystals were used to test for nucleotide binding. On soaking of crystals with ATP/Mg²⁺, additional electron density was found near the anion binding nest of the signature motif [Fig. 3(A)]. The density was modeled satisfactorily with a PP_i/Mg²⁺. Crystallographic statistics are given in Tables I and II. Although one of the phosphates occupies the 164–166 nest, the other interacts with backbone amide 167. Both phosphate moieties and

Figure 3. Structural details of BepA. (A) Signature loop (yellow) with bound PP_i/Mg²⁺ and corresponding F_o-F_c map (with H₁₅₉ side-chain and PP_i/Mg²⁺ omitted for phasing) contoured at 3.5σ. The structure was obtained by soaking a BepA_{tr} crystal overnight in 10 mM ATP and 5 mM MgCl₂. Also shown, in the background, are the two sulfate anions of the BepA₁₀₋₃₀₃ structure (D-chain) bound to two mutually exclusive positions at the N-cap of helix α5, after superposition of the two protein structures. Note the nonmodeled elongation of the electron density of the H₁₅₉ side-chain (see main text). (B) Putative target docking site of BepA₁₀₋₃₀₃, view from the top of Figure 1 with helix α6 running horizontally at the bottom of this figure. Full atom representation with fold cartoon superimposed. The main-chain atoms of the "target dock" in extended conformation [blue in Figs. 1 and 2(A)] of the flap (β2-α4 loop) are shown in ball-and-stick representation. Note, that main-chain amide and carbonyl groups 103 and 105 are fully exposed. The large pocket next to the "target dock" in the middle of the figure accommodates the modified AMP-Tyr side-chain in lbpA(Fic 2), see Figure 2(B). Also shown are the sulfate anions, as in panel (B).

the carboxylic side-chain of E₁₆₃ coordinate the divalent cation. The site is probably not fully occupied as indicated by elevated B-factors of the ligand.

Only when it was reported that the Fic domain of VopS exhibits adenylylation activity,⁶ it became clear

that the observed moiety may represent the side-product of this reaction. In absence of a protein target, ATP may either have been hydrolyzed between the α- and β-phosphate or the AMP moiety may have been transferred to H₁₅₉ with subsequent partial hydrolysis. Indeed, additional density at the imidazole side-chain [Fig. 3(A)] seems to corroborate later scenario.

For further studies and to avoid the problems associated with the uncontrolled processing of BepA that hampered crystal reproduction, a tailored C-terminally His-tagged construct comprising residues 10–303 of BepA (BepA₁₀₋₃₀₃) was expressed and crystallized (Supporting Information Tables S1 and S2). Unfortunately, on soaking of various ATP analogs, no additional density was found. The high salt concentration of the crystallization condition (0.5M NaCl and 1.5M (NH₄)₂SO₄) probably hindered nucleotide binding. Instead, as in the native BepA_{tr} crystals, putative sulphate ions were found within the anion nest 164–166 and the following residue 167 [Fig. 3(A)].

BepA-catalyzed adenylylation

On incubation with α-[³²P]-ATP radioactivity was transferred onto the BepA₁₀₋₃₀₃ fragment [Fig. 4(A)]. This autoadenylation was dependent on the presence of the histidine of the signature motif as evidenced by the negative result obtained with the H159A point mutant, which was properly folded as demonstrated by its crystal structure (not shown). Autoadenylation also occurred with the shortened fragment BepA₁₀₋₂₁₈ [Fig. 4(B)] that was designed to probe the activity of the Fic domain without helix α8 and the following OB-fold. Thus, the activity resides within the canonical Fic fold. Furthermore, the activity was not base-specific. The guanyl- and cytosyl-nucleotides were incorporated with virtually equal efficiency as ATP [Fig. 4(B)]. In the presence of

Table I. Crystallographic Data

	BepA _{tr} ^a		
	SeMet	ATP/Mg ⁺⁺	BepA ₁₀₋₃₀₃ ^b
Wavelength (Å)	0.97935	0.90000	1.0001
Resolution (Å)	2.9	3.0	3.20
Outer resolution shell (Å)	3.06–2.90	3.16–3.00	3.37–3.20
Space group	P2 ₁ 2 ₁ 2 ₁	P2 ₁ 2 ₁ 2 ₁	H32
a (Å)	73.3	72.9	230
b (Å)	84.4	82.9	230
c (Å)	126.7	126.7	309
R _{merge} (%)	10.3 (26.1)	9.8 (33.2)	9.3 (42.1)
Redundancy	5.6 (2.7)	3.4 (3.1)	2.8 (2.6)
I/σ	12.8 (1.9)	6.3 (2.3)	7.3 (2.3)
Completeness (%)	92 (68)	94 (92)	96 (94)
Anomalous completeness (%)	92 (52)	–	–

Data in brackets refer to the outer resolution shell.

^a Full-length BepA GSHMLEM₁-S₅₄₄ proteolytically truncated at C-terminal end during expression/purification.

^b Designed BepA fragment MN₁₀-A₃₀₃HHHHHH.

Table 2. Model refinement

	BepA _{tr} ^a		BepA ₁₀₋₃₀₃ ^b
	SeMet	ATP soak	
Resolution (Å)	30.0–2.90	30.0–3.00	30–3.2
No. of unique reflections	16,307	14,807	46,664
R / R _{free} ^c (%)	21.3/27.4	21.1/27.2	23.9/27.7
Average B (Å ²)	63	66	81
r.m.s. deviation from ideal values			
Bond lengths ^d (Å)	0.011	0.012	0.009
Bond angles ^d (°)	1.1	1.3	1.2
r.m.s.d. NCS related	0.4 ^e	0.4 ^e	0.05–0.2 ^f
C _α -positions (Å)			
Molecules in asymmetric unit	2	2	8
Protein atoms	4449	4458	17898
Pyrophosphate atoms	-	9	-
Water molecules	7	7	-
Ion atoms	6	6	90
Residues in Ramachandran core (%)	98 %	97 %	96 %
Residues in disallowed region (%)	0.4 %	0.5 %	0.9 %
PDB ID	2vy3	2jk8	2vza

^b See Table 1.^c R_{free} calculated with 5 % of the data that were not used for refinement.^d r.m.s.d. from ideal stereochemistry.^e For residues 12–101 and 117–302 after separate superposition.^f r.m.s.d. values for residues 11–101, 117–223, and 239–306 of chain A after individual superpositions on chains B to H, respectively.

HeLa cell extract, adenylyl transfer onto target proteins was evident through the appearance of two additional bands corresponding to sizes of about 40 and 50 kDa on the autoradiograph [Fig. 4(A)]. Because of the different apparent molecular weight from Rho-family GTPases representing the only known protein substrates of Fic domain-containing proteins^{6,7} these proteins likely represent novel Fic domain substrates. Their identification is underway, but beyond the scope of this study.

Model of ternary substrate complex and enzymatic mechanism

Based on the two known binary Fic domain/product complex structures, that is, IbpA(Fic2)/AMP-Y₃₂ and BepA_{tr}/PP_i/Mg²⁺ [Figs. 2(B) and 3(A), see also Supporting Information Fig. S5], it was possible to derive a generic model for the Fic/ATP/Mg²⁺ substrate complex (Fig. 5). For this, we reasoned that the observed PP_i moiety [Fig. 3(A)] most likely maps the β- and α-phosphate subsites of the ATP substrate and not the (original) γ- and β-sites. The assumption

appears reasonable considering that the nest (α-phosphate binding subsite) represents a high-affinity site, which becomes available for PP_i binding on dissociation of the adenylyl moiety. Indeed, after superposition of the protein scaffolds, the PP_i phosphate in the nest and the AMP α-phosphate have a distance of only 1.3 Å (cf. Supporting Information Figs. S5b and S5c). Thus, for model building, the PP_i had to be simply linked to the adenosine moiety of AMP-Tyr and, on the other end, extended by an additional phosphate to yield the triphosphate moiety. Furthermore, the Mg²⁺ cation was repositioned such that it became coordinated by the γ- and α-phosphates and the E163 carboxylate. Finally, the conformation of the R₁₆₇ side-chain was altered to stabilize the γ-phosphate. Figure 5 shows the resulting model of the ternary complex.

Both conserved arginines, R₁₆₇ and R₁₇₀, are predicted to be important for ATP substrate binding. However, in five of the seven known Fic structures (not counting the distantly related AvrB and Doc)

Figure 4. Autoadenylation of BepA fragments and AMP transfer onto HeLa cell extract. BepA variants were incubated with a mixture of hot (α-[³²P]) and cold nucleosyl-tri-phosphate. (A) BepA₁₀₋₃₀₃ (wild-type and mutant H159A) incubated with 20-μM ATP, α-[³²P]-ATP (10 μCi) in the presence or absence of 150 μg of S100 HeLa cell lysate. The control (ctrl) was performed in the absence of BepA. (B) BepA₁₀₋₂₁₈ incubated with 20-μM nucleosyl-tri-phosphate and α-[³²P]-nucleosyl-tri-phosphate (5 μCi); lane 1: ATP, lane 2: GTP, lane 3: CTP.

Figure 5. Model of the ternary complex of BepA(Fic) with ATP/Mg²⁺ and the modifiable tyrosine side-chain (light-green) of the target protein. The model was obtained by combining structural information of the BepAtr/PP_i/Mg²⁺ (2jk8) and the IbpA(Fic2)/cdc42 (3n3v) complexes, see also Supporting Information Figure S5. The structures of the two Fic domains were superimposed, the pyrophosphate moiety of the BepAtr complex extended to a triphosphate and the cation positioned in α -, γ -phosphate bridging location (guided by the structure of ATP in a myosin kinase complex, PDB code: 3lmi). Subsequently, the adenosine moiety of the adenylylated Y₃₂ of the IbpA complex was linked to the α -phosphate and the energy of the structure minimized with program REFMAC²⁷.

the arginines are engaged in salt-bridges to carboxylic side-chains of peripheral α -helices. It is tempting to speculate that on target binding, conformational changes are induced in the Fic domain that cause disruption of these salt-bridges. Such a mechanism would disfavor ATP binding and, thus, futile hydrolysis in the absence of a target. Further kinetic studies are needed to scrutinize this notion. For BepA, the nucleotide base is predicted to bind to a pocket that is structurally equivalent to that of IbpA [Fig. 2(B)]. We were not able to identify hydrophilic residues that would confer base specificity. Indeed the promiscuity observed in the functional assay [Fig. 4(B)] may indicate that there are no H-bonds formed between nucleotide base and enzyme.

The accumulated structural information allows to propose a detailed mechanism for the adenylation reaction catalyzed by Fic domains. Thereby, as proposed before,^{12,16} the histidine of the motif would act as a general base for deprotonation of the tyrosine hydroxyl group. In the model, the hydroxyl group of the tyrosine is in-line with the scissile P _{α} -O_{3 α} phosphoester bond and has distance of 2.8 Å from the phosphorus atom. Nucleophilic attack will generate the penta-coordinated transition state that is probably stabilized by the divalent cation. This would be followed by phosphoester bond cleavage to

yield AMP-tyrosine and pyrophosphate, whereby the PP_i leaving group would again be stabilized by the divalent cation. Nucleotide polymerases, DNA ligases,² and carboxylate:adenylyl transferases^{1,18} operate by the same general mechanism.

Although this mechanism should be representative for most Fic proteins with adenylyl transfer activity, the situation is different in AvrB that seems to be a kinase.¹⁹ Although the modifiable hydroxyl side-chain of the RIN4 is in a similar position as in IbpA(Fic2)/Cdc42, the ADP nucleotide shows a binding mode different to that of ATP in our model. Although the polyphosphate is still bound to the anion binding nest, the direction of the polyphosphate is essentially reversed. As the Thr₁₆₆ side-chain of RIN4 is in a similar position than Y₃₂ of Cdc42, this implies that the γ -phosphate of an ATP substrate could be transferred onto Thr₁₆₆ (after deprotonation by H₂₁₇,¹⁹ a residue not homologous to the usual signature histidine, which is absent in AvrB). Thus, two different but related activities have evolved based on the Fic fold.

In absence of a target, autoadenylation of the Fic domain has been reported for VopS and a eukaryotic Fic domain,¹⁶ which is also observed for BepA₁₀₋₃₀₃ and BepA₁₀₋₂₁₈ (Fig. 4). It is well conceivable that hereby, in a side-reaction, the AMP moiety is transferred onto the active histidine. The orientation of the imidazole side-chain appears suitable for an in-line attack of the N _{ϵ 2} onto the P _{γ} , but the distance is comparatively large (>4.3 Å) suggesting that this would represent an inefficient side-reaction. Autoadenylation could be the first step in a ping-pong mechanism for AMP transfer onto a target. However, consistent with our model, this has been convincingly disproven for VopS by analysis of the enzyme kinetics.²⁰

Target recognition

Fic domain proteins exhibit a rather small conserved core structure. The helical α 4/ α 5 hairpin and the intervening loop seem to be tailored for ATP/Mg²⁺ binding and catalysis of AMP transfer as discussed above. Helices α 2 and α 3 of the core pack against the α 4/ α 5 hairpin by predominantly hydrophobic interactions with the phenylalanine of the motif (F₁₆₁) being part of the hydrophobic core. Although helix α 1 and helices α 6 to α 8 are present in most Fic protein structures, they show considerable variation in location and orientation. Moreover, relative extensions/deletions at the termini or of loop structure are common and it is self-evident that these may participate in target recognition and binding as exemplified by the IbpA(Fic2)/Cdc42 complex structure.¹² Furthermore, as speculated in Ref. ¹², the variability of these peripheral structural elements suggests that Fic structures have evolved to recognize (or rather hijack) distinct targets.

Nevertheless, closer inspection of the accumulated structural information about Fic proteins (Supporting Information Fig. S3) reveals a common feature that may be pertinent for target positioning. All known structures (except for Doc) reveal a β -hairpin or loop structure [“flap” in Figs. 1 and 2(A)] preceding helix α 3. It is found in various orientations (Supporting Information Fig. S3; in BepA_{tr} it is partly disordered), but may, on substrate (ATP and/or protein target) binding, close down onto the active site as commonly observed for kinases to prevent futile ATP hydrolysis. The N-terminal part of the flap may be involved, however, in another important function. This segment, which shows extended backbone conformation in all Fic structures [Supporting Information Fig. S3, see also Fig. 3(C)], has been observed in two cases to form intermolecular antiparallel β -strand interactions with a cognate eukaryotic target protein (IbpA(Fic2)/Cdc42¹² and AvrB/RIN4-peptide complexes¹⁹). Furthermore, in SO2466, this segment form an artifactual interaction of the same kind with a neighboring molecule in the crystal.¹³ While this “target dock” appears well suited to recognize, in a sequence independent manner, target segments in extended conformation, its main role may be to contribute to the precise positioning of the modifiable target residue in the active site of the Fic domain. In fact, residues Y₃₂ of Cdc42 and T₁₆₆ of RIN4 are directly preceding the target dock (residues 33–35 and 167–169, respectively). Such coupling of sequence independent β -strand recognition and productive binding of the susceptible part of the target has also been observed in serine proteinases.²¹

Materials and Methods

Protein expression and crystallization

Full-length BepA with an N-terminal His₆-tag and a thrombin cleavage site was overexpressed in *E. coli* strain BL21 (DE3). Purification was performed on a Ni-NTA column (Qiagen AG, Switzerland). The His₆-tag was cleaved using bovine thrombin (Amersham) at 4°C for 6 h. Uncleaved material was removed by using a second Ni-NTA column. Subsequently, benzamidine sepharose beads (GE Healthcare) were used to remove thrombin from the sample. Pooled fractions were dialyzed against 20 mM TrisHCl (pH 8.0), 500 mM NaCl, and 2 mM β -mercaptoethanol (buffer A) at 4°C, concentrated and loaded onto a Superdex 75 HR 26/60 (GE Healthcare) column. The protein that turned out to be proteolytically cleaved at the C-terminal end was designated BepA_{tr}. The elution volume indicated monomeric protein. Selenomethionine-substituted BepA was expressed using the metabolic inhibition pathway²² and purified the same way as native BepA. Crystals of wild-type and Se-Met protein were obtained by mixing 1- μ L

protein (17 mg/mL) in buffer A with 1.5- μ L 22% polyethylene glycol 4000, 0.1M Tris pH 8.7, 5 mM NiCl₂ reservoir solution.

A BepA construct comprising residues 10–303 and a C-terminal His₆ tag (BepA₁₀₋₃₀₃) was expressed and crystallized by mixing 1- μ L protein (27 mg/mL, in 20 mM Tris pH 8.0, 0.5M NaCl, and 2 mM β -mercaptoethanol) with 1 μ L of 1.5M (NH₄)₂SO₄, 0.1M Tris pH 8.5, and 0.1M Li₂SO₄.

Crystal structure determination

Diffraction data were collected at the Swiss Light Source, Paul Scherrer Institute, Villigen, Switzerland and were processed with MOSFLM/SCALA²³; details are given in Table I. SHELXD²⁴ was used to identify 18 selenium sites of the Se-Met-substituted crystal from the Bijvoet differences determined at the wavelength of the X-ray absorption peak. Phase refinement was performed by SHARP/SOLOMON²⁵ and was followed by twofold averaging and phase extension using DM.²⁶ The model was built at the interactive graphics and refined by using REFMAC²⁷ imposing noncrystallographic twofold symmetry restraints on most of the chain. The resulting model encompasses residues 11–302 with the segment 106–111 and several side-chains undefined by electron density. The refinement statistics is given in Table II.

Activity assay

BepA variants (500 pmol in 20 mM Tris pH 7.5, 100 mM NaCl) were incubated with a mixture of hot (α -[³²P]) and cold nucleosyl-tri-phosphate for 30 min at 30°C. The reaction was stopped by the addition of the SDS sample buffer. Samples were boiled for 5 min at 95°C, subjected to SDS-PAGE and analyzed by autoradiography. S100 HeLa cell lysate was prepared as in Ref. 28.

Conclusions and Outlook

The structure of BepA_{tr} and accompanying data allowed us to derive a generic model for the Fic/ATP/Mg²⁺ substrate complex and to propose a detailed mechanism for the adenylation reaction catalyzed by Fic domains. The identification of the proteins adenylylated by BepA_{tr} in Hela cell extracts will establish a novel class of Fic target proteins different from the known Rho-family GTPases. A subsequent structure/function analysis of the BepA/target complex may allow to prove the proposed adenylation mechanism and, moreover, will address a putative role of the OB fold in target recognition. Together, these data will enhance our understanding of the molecular function of the Fic domain and, moreover, shed light on the role of the BepA effector protein in subverting endothelial cell functions during chronic *B. henselae* infection.

Accession codes

The atomic coordinates and structure factors have been deposited in the Protein Data Bank under accession numbers [2vy3](#) (BepA_{tr}), [2jk8](#) (BepA_{tr}/PP_i/Mg²⁺), and [2vza](#) (BepA₁₀₋₃₀₃).

Acknowledgments

The authors thank the staff of beam-line X06SA of the Swiss Light Source (Villigen, Switzerland) for assistance with data acquisition, Dagmar Klostermeier for preliminary nucleotide binding experiments, and Torsten Schwede and Alex Böhm for stimulating discussions.

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