Mechanistic insights into the activation of bacterial ExoY nucleotidyl cyclase toxins targeting actin-profilin

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Abstract

ExoY virulence factors are members of a family of bacterial nucleotidyl cyclases (NCs) that are activated by specific eukaryotic cofactors and overproduce cyclic purine and pyrimidine nucleotides in host cells. ExoY NC activity is triggered by actin. Here, we investigate ExoY-like toxins that selectively interact with monomeric actin (G-actin) using the ExoY effector domain (Vn-ExoY) of the *Vibrio nigripulchritudo* Multifunctional-Autoprocessing Repeats-in-ToXin (MARTX) toxin. Our biochemical and structural analyses suggest that Vn-ExoY uses profilin-bound G-actin as a physiological cofactor, sequestering this complex and preventing its elongation factor-mediated assembly at the barbed ends of actin filaments. A series of high-resolution crystal structures of nucleotide-free, 3'-deoxy-ATP- or 3'-deoxy-CTP-bound Vn-ExoY, activated by free or profilin-bound G-actin-ATP/-ADP, provide unprecedented mechanistic details of how the active site of NC toxins is sequentially remodelled by cofactor and substrate binding and can accommodate nucleotides of different sizes as substrates. Our structural characterisations, together with mutational data on Vn-ExoY and its distantly-related ExoY from *Pseudomonas*

***aeruginosa*, elucidate important features of their catalytic reaction that will help identify how to inhibit NC toxins.**

INTRODUCTION

ExoY-like nucleotidyl cyclase (NC) toxins are virulence factors produced by several Gram-negative β- and γ-Proteobacteria (Teixeira-Nunes *et al*, 2022). They belong to a family of bacterial NCs that share a structurally-related catalytic NC domain that is inactive in bacteria. When injected into eukaryotic cells, they bind to specific eukaryotic proteins that stimulate their enzymatic activity by several orders of magnitude (Tang & Guo, 2009; Belyy *et al*, 2016). They then become highly active cyclases with broad substrate specificity (Beste *et al*, 2013; Raoux-Barbot *et al*, 2018) and increase the levels of cyclic purine (cAMP, cGMP) and pyrimidine (cCMP, cUMP) nucleotides, thereby interfering with host cell signalling (reviewed in (Ahuja *et al*, 2004; Tang & Guo, 2009; Morrow *et al*, 2017; Seifert, 2017; Teixeira-Nunes *et al.*, 2022)). While the *Bacillus anthracis* edema factor (EF) and *Bordetella pertussis* CyaA toxins are both activated by calmodulin (CaM), the ExoY-like NC toxins use actin (Belyy *et al.*, 2016). CaM-activated toxins have been extensively studied over the last decades. In contrast, the functional characteristics and virulence mechanisms of the ExoY-like NC toxins still need to be clarified.

Exoenzyme Y (Pa-ExoY) is the second most prevalent type III secretion system (T3SS) exotoxin encoded in the genomes of clinical or environmental isolates of *P. aeruginosa* (Feltman *et al*, 2001). Pa-ExoY NC activity in host cells alters the host immune response (He *et al*, 2017; Jeon *et al*, 2017) and leads to the release of cytotoxic amyloids from the pulmonary endothelium, impairing cellular repair after infection (Morrow *et al.*, 2017; Renema *et al*, 2020; Voth *et al*, 2020). In *Vibrio vulnificus* biotype 3, a pathogen that causes severe foodborne and wound infections in humans, the ExoY module of its MARTX toxin (Vv-ExoY) is essential for virulence (Ziolo *et al*, 2014). ExoY NC homologues from different pathogens can potentially exert different cytotoxic effects in infected cells due to differences in their protein sequence, the form of actin they require to become active, and their substrate specificities (Raoux-Barbot *et al.*, 2018). The MARTX Vn-ExoY module of *Vibrio nigripulchritudo*, an emerging pathogen in marine shrimp farming, is representative of the ExoY modules found in *Vibrio* strains. It shares only 38 % sequence similarity with Pa-ExoY. Whereas Pa-ExoY requires actin filaments (F-actin) for maximal activation (Belyy *et al.*, 2016), Vn-ExoY is selectively activated by G-actin *in vitro*. Vn-ExoY, like EF and CyaA (Beckert *et al*, 2014), strongly prefers ATP and can use CTP as substrate, but much less efficiently (Raoux-Barbot *et al.*, 2018). Pa-ExoY, on the other hand, synthesises a wide range of cyclic nucleotide monophosphates (cNMPs) (Beckert *et al.*, 2014) with the following *in vitro*

substrate preference: GTP>ATP≥UTP>CTP (Raoux-Barbot *et al.*, 2018). Recently, cUMP and cCMP have emerged as novel second messengers that activate antiviral immunity in bacteria (Tal *et al*, 2021). On the other hand, very little is known about their role in eukaryotic cells (Seifert & Schirmer, 2022).

Bacterial NC toxins are defined as class II adenylate cyclases (ACs). Their catalytic domain is structurally unrelated to the widely distributed class III adenylyl or guanylyl cyclases (GCs) (Hurley, 1999; Tesmer *et al*, 1999; Steegborn, 2014; Childers & Garcin, 2018; Khannpnavar *et al*, 2020) and may represent a potential drug target against bacterial infections. In their cofactor-bound state, they are several orders of magnitude more active than activated mammalian class III ACs (Tang & Guo, 2009), which use a dimeric catalytic architecture. The molecular basis for the turnover differences between these two AC classes remains to be elucidated. The catalytic domain of NC toxins can be structurally divided into subdomains C_A and C_B, with the catalytic site located in a groove at their interface (Drum *et al*, 2002). EF and CyaA recognise CaM via two flexible regions of C_A that are called switch A (residues 502-551 in EF, 199-274 in CyaA) and switch C (630-659 in EF, 348-364 in CyaA) (Drum *et al.*, 2002; Guo *et al*, 2005; Shen *et al*, 2005). In CaM-bound EF, switch A and C stabilise another key region of C_A, called switch B (578-591 in EF, 299-311 in CyaA). This region contains residues that either directly bind ATP or stabilise catalytic residues in the groove between C_A and C_B (Drum *et al.*, 2002). In the inactive state of EF, switch B is disordered because switch A and C are distant from the catalytic site. Activation of EF or CyaA by CaM does not appear to require a large subdomain movement between C_A and C_B, but rather a CaM-induced movement of switch A and C towards the catalytic site (Tang & Guo, 2009). This in turn stabilises switch B and ATP binding to favour a catalytically competent form of the enzyme. This allosteric activation-by-stabilisation mechanism was recently endorsed for the actin-activated NC toxins. In the first structure of Pa-ExoY crystallised alone after limited in-situ proteolysis, the three switches were not visible in the electron density due to partial proteolysis and/or flexibility (Khanppnavar & Datta, 2018). Recent cryo-EM structures of actin-activated Pa-ExoY and Vv-ExoY revealed that Pa- and Vv-ExoY also use their central switch A and C-terminal switch C to recognise F- and G-actin subunits, respectively (Belyy *et al*, 2021). Molecular dynamics simulations suggested that binding of Pa-ExoY to F-actin stabilises its otherwise flexible switches A and C, thereby stabilising the entire toxin and nucleotide-binding pocket (NBP). Despite these advances, the number of ions required for maximum efficiency, the nature of the catalytic base, or the coordination and role of the residues involved in the various reaction steps are still a matter of debate (Drum *et al.*, 2002; Shen *et al*, 2004; Guo *et al.*, 2005; Shen *et al.*, 2005; Gupta *et al*, 2006; Martinez *et al*, 2009; Mones *et al*, 2013; Jara & Martinez, 2016; Belyy *et al.*, 2021; Teixeira-Nunes *et al.*, 2022). The molecular basis of the NC toxin's pyrimidinyl cyclase activity is also unknown.

Here, using *in vitro* functional studies in actin self-assembly and structural studies, we show that Vn-ExoY targets and is potently activated by profilin-bound G-actin. This interaction also prevents the actin-profilin complex from supporting the assembly of F-actin barbed ends. We present a series of high-resolution structures of Vn-ExoY activated by free or profilin-bound actin-ATP/ADP, in the absence or presence of non-cyclisable ATP or CTP analogues bound to Mg^{2+} or Mn^{2+} ions. The data conclusively demonstrate that NC toxins employ a two-metal-ion mechanism to catalyse the cyclisation reaction of large purine or small pyrimidine nucleotides (with double or single carbon-nitrogen ring bases, respectively). Residues that play an important role in the purinyl and pyrimidinyl cyclase activity of NC toxins are validated in Vn-ExoY and its distantly-related Pa-ExoY.

Results

Vn-ExoY binds to actin monomers with only modest affinity and inhibits actin self-assembly

As a model for ExoY toxins that are selectively activated by G-actin, we used a functional Vn-ExoY$^{3412\text{-}3873}$ effector domain/module corresponding to residues 3412 to 3873 of *V. n.* MARTX (Raoux-Barbot *et al.*, 2018). We first examined the effect of Vn-ExoY on actin-ATP/-ADP self-assembly. To avoid the indirect effects due to ATP depletion by Vn-ExoY AC activity, we used an inactive double mutant K3528M/K3535I, hereafter referred to as Vn-ExoY^{DM} (corresponding to Pa-ExoY$^{K81M/K88I}$) (Raoux-Barbot *et al.*, 2018). Actin polymerisation kinetics were monitored using pyrene-labelled actin, which shows an increase in fluorescence intensity when incorporated into filaments. While inactive Pa-Exo^{K81M} accelerated the rate of actin polymerisation (Fig. 1A) (Belyy *et al.*, 2021), Vn-ExoY^{DM} inhibited the polymerisation rate of both G-actin-ATP-Mg and G-actin-ADP-Mg (Fig. 1B-C). Thus, Vn-ExoY interacts with G-actin-ATP or -ADP in a very different way than Pa-ExoY. To understand the inhibitory effect of Vn-ExoY, we examined the association of free or Vn-ExoY^{DM}-bound G-actin with the barbed (+) or pointed (-) ends of filaments. Spectrin-actin or gelsolin-actin seeds were used to induce elongation at the barbed- and pointed-ends, respectively. Interaction of Vn-ExoY^{DM} with G-actin-ATP-Mg inhibited actin assembly at both ends (Fig. 1D). The dose-dependent effects of Vn-ExoY^{DM} on barbed- and pointed-end elongation rates were consistent with the formation of a 1:1 molar complex between G-actin and Vn-ExoY^{DM} sequestering G-actin with an equilibrium dissociation constant (Kd) of 21 μM (Fig. 1D). To confirm the binding affinity by direct measurement, we performed fluorescence-detected sedimentation velocity experiments using analytical ultracentrifugation (AUC-FDS) with G-actin-ATP labelled with the fluorescent dye Alexa-488 and unlabelled Vn-ExoY fused to Thioredoxin (Trx-Vn-ExoY). This protein fusion, which is more soluble than Vn-ExoY, was preferred for dose-dependent analyses at high concentrations (Fig. 1E). With free labelled G-actin (0.06 μM) bound to Latrunculin-A, an actin-polymerisation inhibitor, the actin sedimentation coefficient

distribution gave a peak centred at an $s_{20,w}$ value of 3.3 S (Fig. 1E, left panel) consistent with a globular actin monomer of 42 kDa. Upon incremental addition of Trx-Vn-ExoY, the sedimentation coefficient distribution of labelled actin shifted to higher s-values (from 3.2 up to 4.7 S), demonstrating the interaction of Vn-ExoY with actin. Peak position and shape were concentration-dependent, and the relative proportion of the larger species in the peaks increased with increasing concentrations of Trx-Vn-ExoY. The titration was consistent with the formation of a 1:1 Vn-ExoY:G-actin complex with a Kd of 11.7 ± 0.9 µM (Fig. 1E, right panel). Thus, Vn-ExoY captures G-actin with only modest affinity to form a complex that sequesters G-actin.

Vn-ExoY binds and is activated by G-actin bound to profilin

We next investigated whether Vn-ExoY could compete with G-actin-binding proteins (G-ABPs) in host cells despite its moderate affinity for G-actin (dos Remedios *et al*, 2003). We examined the *in vitro* AC activity of Vn-ExoY stimulated by G-actin in the presence of three regulatory G-ABPs that display different binding interfaces on G-actin: (i) a chimeric β-thymosin domain of Thymosin-β4 and Ciboulot proteins, which inhibits actin self-assembly by sequestering G-actin (Didry *et al*, 2012), (ii) profilin and (iii) a profilin-like WH2 domain of the Cordon-Bleu protein (Husson *et al*, 2011). The latter two control the unidirectional assembly of actin monomers at the barbed-ends of filaments. At saturating concentrations of these three G-ABPs, only profilin did not interfere with G-actin-induced activation of Vn-ExoY AC activity (Fig. 2A). We then performed AUC-FDS experiments, monitoring the sedimentation behaviour of Alexa-488-labelled-G-actin in the presence of Vn-ExoY, profilin or both proteins. The formation of complexes of increasing size was observed by the shifts from an $s_{20,w}$ value of 3.5 S obtained for the peak of free Alexa488-labelled actin to 4.2 S with profilin (15 kDa), 5.0 S with the larger Trx-Vn-ExoY protein (69.1 kDa), and to a higher value of 5.5 S with both proteins (Fig. 2B), validating the formation of a ternary Vn-ExoY:actin:profilin complex in solution. We next investigated whether the interaction of Vn-ExoY with G-actin was modulated by the simultaneous binding of profilin. We measured the binding strength of Trx-Vn-ExoY^{DM} to fluorescently labelled G-actin-ATP alone or bound to profilin using microscale thermophoresis (MST). Titrations of the interaction by measuring changes in fluorescence (Fig. 2C) or MST signal (Fig. S1) show that Vn-ExoY binds with similar affinity to free or profilin-bound G-actin.

Assembly of actin:profilin at F-actin barbed-ends is inhibited by Vn-ExoY

To understand the effect of Vn-ExoY interaction with actin:profilin on actin self-assembly, we first examined its effect on the spontaneous assembly of actin:profilin at F-actin barbed-ends. We measured the normalised initial rate of barbed-end filament growth in the presence of increasing concentrations

of profilin with or without fixed concentrations of Vn-ExoY. Without Vn-ExoY, barbed-end growth was only modestly inhibited by saturating concentrations of profilin as actin:profilin can associate with and elongate F-actin barbed-ends (Fig. 2D). With Vn-ExoY, the dependence of barbed-end growth on profilin concentration shifted to lower values in a Vn-ExoY dose-dependent manner. This shift was consistent with a decrease in the G-actin pool available for barbed-end elongation due to its sequestration by Vn-ExoY. We next examined the effect of Vn-ExoY on actin:profilin assembly at F-actin barbed-ends regulated by VASP or formin processive barbed-end elongation factors. The latter bind to barbed-ends via an F-actin binding domain (called Formin-Homology domain 2 (FH2) in formins) and favour actin:profilin recruitment to barbed-ends through their adjacent profilin-binding proline-rich motifs (PRMs, which are repeated in formin FH1 domain). Formins or VASP also counteract the barbed-end capping and growth inhibition by capping proteins (CP). The barbed-end growth of F-actin seeds was therefore monitored in the presence of both G-actin:profilin, CP and either the C-terminal FH1-FH2 domains of mouse Dia1 formin (F or mDia1-FH1-FH2, Figure 2E) or full-length human VASP (V, Figure 2F) to approach the physiological regulation of F-actin barbed-ends. When actin (1µM) is saturated with profilin (8 µM), actin nucleation is prevented, and polymerisation does not occur (horizontal dashed grey lines). Under these conditions, the addition of 0.35 nM of spectrin-actin seeds induced rapid elongation of the barbed ends of actin filaments (ascending dotted grey curves). In the presence of 2.1 nM CP, this barbed-end elongation was partially inhibited (black dashed curves), but restored by the addition of 3.2 nM mDia1-FH1-FH2 (Fig. 2E, black curves) or 0.22 µM VASP (Fig. 2F, black curves), as both elongation factors compete with CP at the barbed ends. Further addition of Vn-ExoY at 0.8 or 1.4 µM to mDia1-FH1-FH2 or VASP in the presence of CP inhibited the barbed-end growth from actin:profilin induced by the two elongation factors (blue curves). These inhibitory effects were independent of Vn-ExoY AC activity for the duration of the kinetic experiments (Fig. S2). Thus, the interaction of Vn-ExoY with G-actin:profilin inhibits the spontaneous or VASP-/formin-mediated assembly of this complex at the most dynamic ends of F-actin.

Crystal structures of nucleotide-free and 3'-deoxy-ATP-bound Vn-ExoY in complex with actin-ATP/ADP:profilin or actin-ATP

Next, we investigated the structural mechanism of Vn-ExoY activation by free or profilin-bound G-actin. To improve the crystallisation strategy (see Supplementary Information), we designed a chimeric protein containing three fused functional domains: Vn-ExoY extended at the C-terminus (Vn-ExoY$^{3455-3896}$, either wild-type or with K3528M/K3535I), a short profilin-binding proline-rich motif (PRM) and profilin (Fig. 3A, VnE/VnE^{DM}-PRM-Prof). The chimera sequestered G-actin with approximately 100-fold higher affinity than the Vn-ExoY$^{3412-3873}$ module and exhibited strong actin-dependent AC activity

at low G-actin concentrations (Fig. 3B-C). Thus, the actin-binding regions in Vn-ExoY and profilin appear to be fully functional in this chimera. Using this chimera (wild-type or double-mutant), we solved two crystal structures at 1.7 Å resolution: (i) VnE^{DM}-PRM-Prof bound to actin-ADP with latrunculin B (LatB), and (ii) VnE-PRM-Prof bound to actin-ADP without LatB (Fig. 3D, Table S1). In these structures, profilin is bound to actin-ADP, the PRM to profilin, Vn-ExoY to actin, while residues 3863-3895 connecting the C-terminus of Vn-ExoY to the PRM are disordered (Fig. 3D, Table S1). Vn-ExoY contacts actin-ADP via its switch A and C, but only switch C is fully ordered. The Vn-ExoY switch A disorder (residues 3682-3727) is close to the LatB-binding site in actin. However, solving the structure without LatB only slightly reduces the switch A disorder. In the latter structure, the NBP of Vn-ExoY is further stabilised by a sulphate ion (Fig. 3D, VnE-PRM-Prof-SO_4^{2-}:actin-ADP). On the basis of these structural data, we designed a minimal functional Vn-ExoY module (residues 3455-3863) by removing the C-terminal disordered residues 3864-3895 (Fig. 3A). This Vn-ExoY module (shortened by 32 C-terminal residues) shows similar catalytic activity to our original Vn-ExoY$^{3412-3873}$ construct and allowed the determination of two additional crystal structures: (i) nucleotide-free Vn-ExoY bound to actin-ATP-LatB and profilin, with a sulphate ion in Vn-ExoY NBP (Fig. 3E, Vn-ExoY-SO_4^{2-}:actin-ATP-LatB:profilin), and (ii) Vn-ExoY bound to actin-ATP-LatB with its NBP filled by a non-cyclisable ATP analogue 3'-deoxy-ATP (3'dATP, Figure 3F), at 1.7 and 2.1 Å resolution, respectively. The Vn-ExoY-SO_4^{2-}:actin-ATP-LatB:profilin structure is very similar to the structures of the VnE/VnE^{DM}-PRM-Prof chimera bound to actin-ADP, with the Vn-ExoY switch A similarly disordered. In contrast, the Vn-ExoY-3'dATP-2Mg^{2+}:actin-ATP-LatB structure co-crystallised with 3'dATP reveals a more ordered Vn-ExoY:actin interface and a distinct conformational state of Vn-ExoY NBP that is more competent for catalysis, as described below.

In the G-/F-actin-bound state, the switch-C of ExoYs ensures stable interactions while other regions remain dynamic

Vn-ExoY undergoes significant structural rearrangements at both the C_A and C_B subdomains between its nucleotide-free conformation bound to actin-ATP/ADP and profilin, and its 3'dATP-bound conformation in complex with actin-ATP (Fig. 4A). At the Vn-ExoY:actin interface, none of the buried contacts of the Vn-ExoY switch C move, except for L3827, which is relocated by the remodelling of switch A (Fig. 4A-B). Thus, switch C appears to be essential for initiating the interaction and stabilising Vn-ExoY on actin. In the absence of 3'dATP, most of switch A is too flexible to be modelled, and the switch A portions that are ordered in the absence of ligand undergo profound rearrangements upon 3'dATP-binding (up to 10 Å on D3682 and K3683 Cα atoms, Figure 4A). Subsequently, the letters after the residue numbers are used to distinguish residues of different proteins or functional regions (e.g. $^{VnE-}$

^{CA} refers to residues belonging to the Vn-ExoY subdomain C_A). The different conformations of Vn-ExoY bound to actin/actin:profilin with or without a nucleotide substrate analogue allow us to define the mobile "switch" regions as follows: Vn-ExoY switch A as residues Y3675-V3727$^{VnE\text{-}Switch\text{-}A}$ (I222-N264$^{PaE\text{-}Switch\text{-}A}$, UniProt AC Q9I1S4_PSEAE), switch B as H3753-A3770$^{VnE\text{-}Switch\text{-}B}$ (H291-A308$^{PaE\text{-}Switch\text{-}B}$), switch C as I3819-L3863$^{VnE\text{-}Switch\text{-}C}$ (K347-V378$^{PaE\text{-}Switch\text{-}C}$). Other important conformational changes near the interface occur in the parts of switch C in contact with switch A and B, and in the parts of switch B in contact with switch A and C (Fig. 4A-B). These regions show high B-factors in all the complexes with nucleotide-free Vn-ExoY (Fig. 4D-1). Near the interface, the C_B subdomain of Vn-ExoY undergoes the most drastic global rearrangement with a large 25° rotation as a rigid body around the hinge regions 3532-3533^{VnE} and 3662-3663^{VnE}. It thus acts as a lid (hereafter referred to as C_B/LID, residues K3533-M3662$^{VnE\text{-}LID}$, corresponding to K86-M209$^{PaE\text{-}LID}$ in Pa-ExoY) that closes on C_A to stabilise 3'dATP binding (Fig. 4A-B, movie S1A-B). The conformational changes induced by 3'dATP binding bring several regions of the Vn-ExoY C_A subdomain closer to actin, increasing the buried solvent-accessible area (BSA) of switch A from 338 to 707 Å^2, reaching the BSA value of switch C (810 and 766 Å^2, respectively). Vn-ExoY binds to actin over a large surface area, with switch A binding primarily to actin subdomains 2 and 4 and switch C binding to actin subdomains 1 and 3 (Fig. 4C).

To validate the importance of switch C for proper positioning of ExoY on actin, we mutated conserved buried residues of switch C that remain well anchored and stable in the complexes with or without 3'dATP. Actin D25 (D25^{Actin}) forms a buried salt bridge at the interface core with the R3824$^{VnE\text{-}switch\text{-}C}$ side chain (distances of 2.7 and 3.2 Å between N-O atom pairs, Figure 3D-F, 4B-C) and was identified as a critical residue for Pa- and Vn-ExoY activation by F- and G-actin, respectively (Belyy *et al.*, 2016). The R3824A$^{VnE\text{-}switch\text{-}C}$ mutation alone results in a 130-fold reduction in Vn-ExoY G-actin stimulated AC activity (Fig. 5A). The extreme C-terminus of Pa-ExoY is also critical for its binding to F-actin (Belyy *et al*, 2018). The corresponding region in Vn-ExoY is located at the edge of the Vn-ExoY:actin interface, where the extreme C-terminal amphipathic helix of Vn-ExoY (residues 3847-3863$^{VnE\text{-}switch\text{-}C}$) binds into the hydrophobic cleft between actin subdomains 1 and 3. Mutation of the most buried hydrophobic residues of this amphipathic helix to alanine results in a 240-fold reduction in its AC activity (Fig. 5A). Similar interactions occur in the switch C of F-actin-bound Pa-ExoY (Belyy *et al.*, 2021). Three basic amino-acids of Pa-ExoY near D25^{Actin} may play the same role as R3824$^{VnE\text{-}switch\text{-}C}$: K347$^{PaE\text{-}switch\text{-}C}$, H352$^{PaE\text{-}switch\text{-}C}$ and R364$^{PaE\text{-}switch\text{-}C}$. While the R364A mutation in Pa-ExoY switch C had almost no effect on its F-actin-stimulated GC activity, the K347A and H352A mutations caused a ~15- and ~8-fold decrease, respectively (Fig. 5C), indicating that these two amino-acids together function as R3824$^{VnE\text{-}switch\text{-}C}$. On the other hand, changing the three bulky hydrophobic side chains of the C-terminal amphipathic helix of Pa-ExoY (i.e. F367-L371-F374, Figure 5B) into alanine

caused a ~12,000-fold reduction in F-actin stimulated GC activity (Fig. 5C). These residues in the switch C of Vn-ExoY or Pa-ExoY are conserved in most of their counterparts in other β- and γ-Proteobacteria (Fig. 5D). Thus, during the transition of these enzymes to their catalytic conformation, their complex with G-/F-actin is first stably docked to their cofactor at their switch-C, while other regions remain dynamic to regulate additional functional rearrangements.

Significance of switch A and C_B/LID closure in stabilising nucleotide and Mg^{2+}

Except for a difference in the structural topology of the proteins (Fig. S3), the overall structure of Vn-ExoY bound to actin and 3'dATP resembles *V. vulnificus* Vv-ExoY bound to actin:profilin (Belyy *et al.*, 2021) (Fig. S4A). In the 3.9-Å resolution cryo-EM structure, the 3'dATP ligand used for the preparation of the complex could not be modelled in Vv-ExoY. In both structures, switch A adopts a similar conformation between actin subdomains 2 and 4. This interaction primarily prevents Vn- and Vv-ExoY from binding along the side of F-actin. This explains their G-actin specificity, whereas the orientation of Pa-ExoY switch A bound to F-actin differs (Belyy *et al.*, 2021) (Fig. S4B). Furthermore, the positioning of the Vn-ExoY switch A on G-actin prevents the association of Vn-ExoY:G-actin or Vn-ExoY:G-actin:profilin complexes with F-actin barbed-ends (Fig. S5A) and is responsible for the inhibition seen in Figure 1D, 2D-F. Switch A is also the region with the most divergent folding at the toxin:cofactor interface between G/F-actin-bound ExoYs and CaM-bound EF or CyaA (Figs. S5B-C). Thus, this region appears to be evolutionarily essential for the cofactor specificity of NC toxins.

Our different structures reveal two major consecutive conformational changes occurring at the catalytic site of NC toxins. In the free/inactive Pa-ExoY structure (Khanppnavar & Datta, 2018), the A, B, and C switches were not visible as partially degraded and/or flexible. In our nucleotide-free structures of Vn-ExoY bound to G-actin-ATP/-ADP and profilin (Fig. 3D-E), the Vn-ExoY NBP is still only partially stabilised by cofactor binding (Fig. 4D-1), showing that cofactor binding is not sufficient to fully stabilise the NBP and catalytic residues of NC toxins, as previously suggested (Drum *et al.*, 2002; Tang & Guo, 2009; Belyy *et al.*, 2021). 42 to 46 of the 53 residues from switch-A are still disordered. A SO_4^{2-} ion is bound to the Vn-ExoY NBP via residues ($K3528^{VnE\text{-}CA}$, $K3535^{VnE\text{-}LID}$, $S3536^{VnE\text{-}LID}$, $K3554^{VnE\text{-}LID}$) that coordinate the γ- and β-phosphates of 3'dATP when this ligand is bound (Fig. 4D-2). Binding of the ATP substrate and Mg^{2+} ion in the Vn-ExoY NBP requires rearrangement of switch B and the supporting part of switch C (Fig. 4D-3). To reach its most favourable binding position, the nucleotide must move ~4 Å (distance between the SO_4^{2-} ion and 3'dATP γ-phosphate) through the LID closure (Fig. 4D-3). Structural rearrangements in the region of switch C, which supports switch B, are accompanied by stabilisation of switch A and the substrate analogue (Fig. 4D-4,4D-5). In the final stage, switch B and C are well-ordered, while the entire switch A is stabilised on G-actin (Fig. 4D-5). In the

Vn-ExoY-3'dATP-2Mg^{2+}:actin-ATP-LatB structure, switch A ultimately contributes to the stabilisation of the nucleotide base through interactions with M3726$^{VnE\text{-}switch\text{-}A}$.

Catalytic mechanism of ExoY NC toxins activated by G- or F-actin with purine nucleotides

In the known cofactor:toxin structures, the nucleotide ligands adopt different positions and conformations in the toxin catalytic pocket, variably coordinated by one or two metal ions (Fig. S6-7). The Pa-ExoY-bound 3'dGTP is coordinated by a single metal ion in the F-actin-activated Pa-ExoY structure (Belyy *et al.*, 2021). In the structure of Vn-ExoY bound to actin and 3'dATP, the electronic density and coordination geometry suggest that 3'dATP is coordinated by two Mg^{2+} ions. To unambiguously distinguish metal ions from water, we replaced Mg^{2+} with Mn^{2+} ions and collected an anomalous data set at the manganese absorption edge (Supplementary Materials). The anomalous difference Fourier map confirms that two Mn^{2+} ions are bound to 3'dATP at the positions occupied by the Mg^{2+} ions, with no significant changes elsewhere (Fig. 6B). Mg^{2+}_{A} is pentacoordinated in a square pyramidal geometry by two aspartates D3665$^{VnE\text{-}CA}$ and D3667$^{VnE\text{-}CA}$, and H3753$^{VnE\text{-}switch\text{-}B}$, which are invariant in all NC toxins (Fig. 6A), an α-phosphate oxygen of 3'dATP, and a water molecule. Mg^{2+}_{B} is octahedrally hexacoordinated by the same two aspartates (D3665/D3667$^{VnE\text{-}CA}$), one oxygen from each of the three phosphates of 3'dATP and a water molecule.

Figure 6A summarises the main interactions observed with 3'dATP in the Vn-ExoY-3'dATP-2*Mg^{2+}:actin-ATP-LatB structure, extrapolated to the natural substrate ATP. The residues of Vn-ExoY that coordinate the nucleotide and metal-ions are indicated in coloured boxes in the first inner shell. The corresponding residues in the Pa-ExoY, EF, and CyaA sequences are in the next outer shells. 9 out of the 14 substrate and Mg^{2+} ion binding residues belong to regions of the enzyme that are flexible and significantly rearranged and stabilised upon binding of the cofactor, nucleotide substrate and metals. This explains the low basal activity of the free enzyme (Raoux-Barbot *et al.*, 2018). All side-chain contacts are provided by residues that are invariant or similar in Pa-ExoY, EF, and CyaA, and most interactions are conserved in CaM-activated EF bound to 3'dATP with a Yb^{3+} ion and a closed LID (PDB 1k90) (Drum *et al.*, 2002) (Fig. S7D).

To assess the relevance of the observed interactions with the ATP analogue, some of the conserved residues involved were mutated in Vn- and Pa-ExoY and the effect on their AC and GC activity was investigated. H351$^{EF\text{-}LID}$ in EF has been proposed to play a role in the 3'OH deprotonation, either by accepting the 3'OH proton (general base), or by facilitating the presence of OH^- ions near the ATP 3'OH group (Drum *et al.*, 2002; Guo *et al*, 2004; Shen *et al.*, 2005; Gupta *et al.*, 2006; Mones *et al.*, 2013; Jara & Martinez, 2016). H351$^{EF\text{-}LID}$ is conserved in CyaA, but in ExoYs it corresponds to a lysine: K3533$^{VnE\text{-}LID}$ or K86$^{PaE\text{-}LID}$ (Fig. 6A). In the active site of Vn-ExoY bound to actin, 3'dATP and

2 Mg^{2+} or 2 Mn^{2+}, the K3533$^{VnE\text{-}LID}$ side chain is too distant to potentially coordinate the ATP 3'OH moiety if the latter were present. K3533Q$^{VnE\text{-}LID}$ or K86Q$^{PaE\text{-}LID}$ mutations only decreased G/F-actin-activated AC and GC activity by 14- and 12-fold, respectively (Fig. 6C-D), further suggesting that these residues are unlikely to act as a general base. Next, we examined K3528$^{VnE\text{-}CA}$ and K3535$^{VnE\text{-}LID}$, which are close to the bridging O3α and non-bridging O2α, O1β and O2γ oxygens of 3'dATP (Fig. 6A). Both side chains are ~3.4 Å from O3α. K3528$^{VnE\text{-}CA}$ is closer to O2α and O2γ (2.8-2.9 Å) than K3535$^{VnE\text{-}LID}$, and K3535$^{VnE\text{-}LID}$ is closer to O1β (3.3 Å). The catalytic activity of Vn- and Pa-ExoY was essentially abolished (at least ~23,000-fold reduction) by the K3528Q$^{VnE\text{-}CA}$ and the corresponding K81Q$^{PaE\text{-}CA}$ mutations in Pa-ExoY. The K3535Q$^{VnE\text{-}LID}$ or the equivalent K88Q$^{PaE\text{-}LID}$ mutations resulted in a 250- and 26-fold decrease in G-/F-actin-induced Vn-ExoY AC or Pa-ExoY GC activity, respectively (Fig. 6C-D). The invariant H3753$^{VnE\text{-}switch\text{-}B}$, which coordinates $Mg^{2+}{}_{A}$ and stabilises the purine base via a π-π stacking with its imidazole ring, is specific to NC toxins. $Mg^{2+}{}_{A}$ may be particularly important in the NBP of activated mammalian ACs to coordinate and polarise the 3'OH of ATP, facilitating its deprotonation as a first step in the cyclisation reaction (Tesmer *et al.*, 1999; Steegborn, 2014; Hahn *et al*, 2015). The H3753Q$^{VnE\text{-}switch\text{-}B}$ or equivalent H291Q$^{PaE\text{-}switch\text{-}B}$ mutations greatly reduced the catalytic activity of the toxins (~13,000-fold reduction), suggesting a critical role for the switch-B invariant histidine and $Mg^{2+}{}_{A}$ in the catalysis of NC toxins.

Structural basis for the pyrimidinyl cyclase activity of ExoYs

The activity of NC toxins as pyrimidine nucleotide cyclases was identified some time ago, both *in vitro* (Gottle *et al*, 2010; Raoux-Barbot *et al.*, 2018) and in eukaryotic cells (Beste *et al.*, 2013; Beckert *et al.*, 2014), but remains poorly characterised. Among the pyrimidine nucleotides, Vn-ExoY, EF or CyaA all primarily use CTP as a substrate, whereas Pa-ExoY prefers UTP but can also use CTP. To determine how activated NC toxins can also use small pyrimidine nucleotides as substrates, we solved the structure of Vn-ExoY bound to 3'dCTP in complex with actin-ADP at 2.2 Å resolution (Vn-ExoY-3'dCTP-2*Mn^{2+}:actin-ADP-LatB). This is the first structure of an activated NC enzyme to be determined in association with a pyrimidine nucleotide substrate analogue. The overall conformation of Vn-ExoY in this structure is identical to those found in the complexes with actin-ATP, 3'dATP and two Mg^{2+} or Mn^{2+}. As with 3'dATP, the LID of Vn-ExoY is closed on 3'dCTP and two metal ions coordinate the phosphate moiety of 3'dCTP. In the NBP of Vn-ExoY, 3'dCTP is stabilised by the same residues and types of interactions as 3'dATP (Fig. 7A). The main differences are in the interactions with the nucleotide ribose and base moieties, which are stabilised by fewer and weaker hydrogen bonds and framed by fewer hydrophobic interactions with 3'dCTP than with 3'dATP. With 3'dATP, the ribose is stabilised by L3530$^{VnE\text{-}CA}$ hydrophobic interactions and hydrogen-bonded by K3533$^{VnE\text{-}LID}$ and

N3759$^{VnE\text{-}switch\text{-}B}$ side-chains (Fig. 6A). In the activated structures of Vn-ExoY and tmAC (Tesmer *et al.*, 1999) with an NBP closed on an ATP analogue, N3759$^{VnE\text{-}switch\text{-}B}$ and N1025$^{tmAC\text{-}IIC2}$ similarly hydrogen-bond the O4' oxygen of the nucleotide ribose ring (Fig. 6A, 6F). Just as mutations at N1025$^{tmAC\text{-}IIC2}$ reduce tmAC kcat by 30-100 (Yan *et al*, 1997), mutations at N583$^{EF\text{-}switch\text{-}B}$ in EF (equivalent to N3759$^{VnE\text{-}switch\text{-}B}$) reduce EF catalytic activity by at least two orders of magnitude (Drum *et al.*, 2002). These data highlight the importance of hydrogen bonding with the nucleotide ribose for correct positioning during class II and III AC catalysis. With 3'dCTP, electrostatic interactions with the nucleotide ribose switch to a single, more labile hydrogen bond between the cytidine ribose ring O4'-oxygen and a water molecule coordinated by D3756$^{VnE\text{-}switch\text{-}B}$ or N3759$^{VnE\text{-}switch\text{-}B}$. The cytosine base is hydrogen-bonded by M3726$^{VnE\text{-}switch\text{-}A}$ and A3755$^{VnE\text{-}switch\text{-}B}$ as for the adenine base. However, these residues only coordinate the N4 atom of cytosine. For 3'dATP, they stabilise both the N1 and N6 atoms of adenine through shorter hydrogen bonds. Similarly, in the Pa-ExoY:3'dGTP-1*Mg^{2+}:F-actin structure, the N1, N2, and O6 guanine atoms are hydrogen-bonded by the Ser292$^{PaE\text{-}switch\text{-}B}$, H291$^{PaE\text{-}switch\text{-}B}$, and Glu258$^{PaE\text{-}switch\text{-}A}$ side chains, respectively (Fig. S7C). The pyrimidine base is otherwise surrounded by similar hydrophobic interactions to the purine base of 3'dATP. However, it lacks Pro3760 side-chain interaction with imidazole ring of 3'dATP purine base on solvent accessible side. On the triphosphate moiety of 3'dCTP, the phosphates and two metal ions are tightly coordinated and less solvent-accessible than the nucleoside moiety in Vn-ExoY NBP, which is very similar to 3'dATP.

The mutations analysed above for Vn-ExoY AC or Pa-ExoY GC activity were reconsidered and their effect on their cytidylyl or uridylyl cyclase (CC/UC) activity was examined to determine whether the conserved interactions with the nucleotide triphosphate moiety play a similar role in the purinyl and pyrimidinyl cyclase activity of ExoYs. The mutations with the greatest effect on Vn-ExoY CC or Pa-ExoY UC activity (150- to 480-fold reduction) are those that also most affect their AC or GC activity, namely K3528Q$^{VnE\text{-}CA}$ or K81Q$^{PaE\text{-}CA}$ and H3753Q$^{VnE\text{-}switch\text{-}B}$ or H291Q$^{PaE\text{-}switch\text{-}B}$, respectively (Fig. 7B-C). As with 3'dATP, the K3528$^{VnE\text{-}CA}$ side chain hydrogen-bonds the α, β, and γ phosphate oxygens of 3'dCTP, and the H3753$^{VnE\text{-}switch\text{-}B}$ side chain stabilises both $Mg^{2+}{}_{A}$ via a hydrogen bond and the cytosine base via a π-π stacking with its imidazole ring. Taken together, these results demonstrate that NC toxins use a similar two-metal-ion catalytic mechanism with purine and pyrimidine nucleotide substrates, relying on the close coordination of their triphosphate moiety for catalysis.

Discussion

Here, we have used the MARTX Vn-ExoY module to explore the functional specificities of ExoY-like toxins that selectively interact with G-actin (Raoux-Barbot *et al.*, 2018). Our data show that Vn-ExoY's modest affinity for free G-actin (Fig. 1D-E, Kd~12 µM compared to a Kd of ~0.2-20 nM

for CyaA or EF binding to CaM (Shen *et al*, 2002)) is compensated by its ability to interact with and be efficiently activated by G-actin bound to profilin (Fig. 2A-C). Vn-ExoY is unable to bind to G-actin in complex with other common G-actin binding proteins such as Thymosin-β4, WH2 domains (Fig. 2A) or actin-depolymerising factor (ADF)/cofilin due to overlapping binding interfaces between actin subdomains 1 and 3 (Fig. S9). The G-actin:profilin complex is abundant and important for supporting filament assembly at barbed-ends in eukaryotic cells (dos Remedios *et al.*, 2003; Pimm *et al*, 2021). It is therefore probably the major physiological form of G-actin that is recognised and used as a cofactor by Vn-ExoY and closely-related ExoYs in eukaryotic cells. Our structural bioinformatics analysis suggests that many ExoY-like homologues from different γ-proteobacteria are indeed structurally-related to Vn-ExoY (Fig. 5D). Vn-ExoY interaction with G-actin:profilin inhibits both spontaneous and formin- or VASP-mediated association of G-actin:profilin complexes with F-actin barbed-ends *in vitro*, independent of the toxin's NC activity (Fig. 2D-F, S2). However, as shown by the multipartite interactions of our actin-bound VnE-PRM-Prof chimera, the ternary complex does not prevent profilin from interacting with PRM segments (Fig. 3D). Further studies are required to determine whether the formation of such a Vn-ExoY:G-actin:profilin abortive ternary complex for barbed-end elongation may also contribute to Vn-ExoY cytotoxicity in host cells independently of its NC activity. Several observations suggest that Pa-ExoY may also act on eukaryotic target cells independently of its enzymatic activity (Belyy *et al.*, 2016; Mancl *et al*, 2020; Silistre *et al*, 2021). The interaction of Vn-ExoY with actin:profilin may be particularly important in determining subcellular localisation and activation at sites of active actin cytoskeleton remodelling (Fig. 8). At these sites, G-actin:profilin and elongation-promoting factors play a prominent role together in fine-tuning the coordination and competition of F-actin networks (Suarez *et al*, 2015; Skruber *et al*, 2020). This fine-tuning of actin dynamics is coordinated by a large number of multidomain ABPs and is tightly associated with cAMP signalling in important cellular processes, including cell adhesion and T-cell migration (Howe, 2004; Yamashita *et al*, 2011; Simao *et al*, 2021; Teixeira-Nunes *et al.*, 2022). VASP activity is regulated by PKA and PKG (Aszodi *et al*, 1999) and is important for immune function (Estin *et al*, 2017). Recruitment of the abortive ternary complex Vn-ExoY:actin:profilin by the PRMs of multidomain ABPs may be of interest to pathogens to confine Vn-ExoY-like toxin activity to these discrete sites where signalling and cytoskeletal remodelling are intense and highly intertwined. These elements provide insight into why ExoY toxins target actin and not calmodulin as canonical NC toxins do.

Our structural data show that Vn-ExoY switch C interactions at the Vn-ExoY:actin interface remain stable with different conformations of the LID/C_B subdomain and switch A (Fig. 3D-F, 4A-B), thus firmly docking the toxin to actin. Our mutational analysis reveals two hotspots in the switch C of Vn- and Pa-ExoY, whose mutation almost completely abolishes their G- or F-actin-dependent NC

activity (Fig. 5). These sites could be hits in the search for ways to inhibit ExoY toxins. In contrast, switch A is only barely stabilised by actin in the absence of substrate (Fig. 3D-E, 4D). EF and CyaA, on the other hand, have a switch A that is already fully folded on CaM in their nucleotide-free, cofactor-bound state (Drum *et al.*, 2002; Guo *et al.*, 2005). However, their C_B/LID subdomain can also adopt different positions in isolated CyaA (Malliavin, 2017) or between different crystal structures of CaM-bound EF (Fig. 6E, S8). Many crystal structures of CaM-activated EF or CyaA with nucleotide ligands in the active site were first crystallised without substrate analogues or reaction products and then incubated with these ligands. Crystal packing in these structures may have prevented complete rearrangement of the NBP and LID/C_B subdomain closure. As a result, and due to the intrinsic flexibility of the LID/C_B subdomain, both substrate analogues and metal ions occupy quite different positions in the NBP of CaM-bound EF or CyaA (Fig. S6-8) (Teixeira-Nunes *et al.*, 2022). These structures may correspond to intermediate stages of LID/C_B movement and substrate entry. Cofactor binding thus initiates allosteric stabilisation of switch B in NC toxins by repositioning the central switch A and the C-terminal switch C towards switch B, as previously reported (Drum *et al.*, 2002; Tang & Guo, 2009; Belyy *et al.*, 2021). In the ExoY NC toxins, however, complete stabilisation of switch A on actin also requires substrate binding and C_B/LID closure. Switch A is involved in the correct positioning of the substrate by directly coordinating the purine or pyrimidine base of the nucleotides in all cofactor-bound NC toxins (Fig. 6A, 7A, S7). In both the inactive conformation of free Pa-ExoY (Khanppnavar & Datta, 2018) and the nucleotide-free conformations of actin:profilin-bound Vn-ExoY (Fig. 3D-E), $H291^{PaE\text{-}switch\text{-}B}$ and $H3753^{VnE\text{-}switch\text{-}B}$, respectively, are stabilised away from the NBP by a salt bridge with $D3736^{VnE}$/$D274^{PaE}$, which are located near the switch A (Fig. 4D). After cofactor binding, substrate binding in ExoYs induces the flipping of $H3753^{VnE\text{-}switch\text{-}B}$/$H291^{PaE\text{-}switch\text{-}B}$ towards the NBP. This invariant histidine of NC toxin switch B assists in the correct positioning of the catalytic Mg^{2+}_A on the two invariant aspartates ($D3665D3667^{VnE\text{-}CA}$ or $D212D214^{PaE\text{-}CA}$) with purine or pyrimidine nucleotide substrate analogues (Fig. 6A-B, 7A). For all NC toxins, substrate binding therefore ultimately facilitates the C_B/LID closure.

Our data demonstrate unambiguously that Vn-ExoY uses a two-metal-ion-assisted catalytic mechanism with purine and pyrimidine nucleotides. Given the sequence similarity and structural conservation, this is most likely to be the case for all bacterial NC toxins after LID/C_B closure (Fig. 6A-B, 7A, S7). Interestingly, the coordination of metal ions and residues of the Vn-ExoY NBP with the ATP-analogue is also closer to that observed in the structures of activated class III ACs (Tesmer *et al.*, 1999) than to that previously observed in the NBPs of cofactor-activated NC toxins (Fig. 6A, 6F, S7). The active site of transmembrane or soluble class III ACs (tmAC/sAC) is located at the interface of their two homologous catalytic domains (termed C1 and C2 in tmAC, Figure 6F). It has also been

proposed to undergo an open-closed transition during catalysis. In the case of tmAC/sAC, however, the exact role of the open-closed transition of their dimeric NBP and the identification of the dynamic active site processes during substrate binding and turnover remain to be clarified (Steegborn, 2014). In the closed NBP of all ACs, the correct positioning of the different ATP moieties is essential for catalysis. This is shown by the strong effect of mutations targeting N1025$^{\text{tmAC-IIC2}}$ in tmAC (Yan *et al.*, 1997; Tesmer *et al.*, 1999) or N583$^{\text{EF-switch-B}}$ in EF (Drum *et al.*, 2002), which corresponds to N3759$^{\text{VnE-switch-B}}$ in Vn-ExoY. These conserved asparagines similarly hydrogen-bond the ribose ring O4' oxygen of the ATP analogue for catalysis (Fig. 6A, 6F). On the phosphate and 3'OH side, the most probable reaction sequence as well as the key interactions during each step were investigated by computational studies for tmAC with a closed ATP-binding NBP (Hahn *et al.*, 2015) and for EF with an open or nearly-closed ATP-binding NBP (using PDB 1XFV/1SK6) (Mones *et al.*, 2013). The reaction model for tmAC better fits the numerous and tight interactions around 3'dATP and metal ions observed in the closed NBP of activated Vn-ExoY. The proposed mechanism for tmAC is a substrate-assisted general base catalysis, where several residues of the enzyme appear to be particularly important with metal-ion cofactors for lowering the two highest energy barriers of the reaction. First, K1065$^{\text{tmAC-IIC2}}$, $Mg^{2+}{}_{A}$, $Mg^{2+}{}_{B}$ and the two conserved D396D440$^{\text{tmAC-VC1}}$ anchoring metal ions are critical for jointly facilitating the proton transfer from the ribosyl 3′O to a γ-phosphate oxygen (Hahn *et al.*, 2015). R1029$^{\text{tmAC-IIC2}}$ is then critical for the concerted phosphoryl transfer step in which K1065$^{\text{tmAC-IIC2}}$ is also involved. These interactions belong to C1 and C2 structural elements that are distant in the open NBP of tmAC (Tesmer *et al.*, 1999). Similarly, in NC toxins, the NBP closure brings together ATP/CTP- and metal-ion-coordinating residues belonging to four distinct regions, which are distant from each other in the absence of cofactor and substrate. Three of these ATP/CTP- and metal-ion coordination regions are intrinsically flexible. Their positioning and stabilisation depend on both cofactor and substrate binding for switch A and B, and substrate binding for C_B/LID (Fig. 6A, 7A). Substrate-induced C_B/LID closure after activation ultimately reduces the degrees of freedom of the nucleotide substrate and metal ions in the NBP (Fig. S7) to promote catalysis. Our mutational analysis in Vn-ExoY and the distantly-related Pa-ExoY identifies the common elements of the NC toxin active sites that contribute to catalysing the limiting steps in the cyclisation reaction with substrates of different sizes. The analysis pinpoints K3528$^{\text{VnE-CA}}$/K81$^{\text{PaE-CA}}$ and H3753$^{\text{VnE-switch-B}}$/H291$^{\text{PaE-switch-B}}$. Their mutations to Gln (to preserve polar interactions with ATP or Mg^{2+}) strongly alter the AC/GC and CC/UC activity in Vn-/Pa-ExoY, respectively (Fig. 6C-D, 7B-C). These residues are invariant in EF or CyaA and the same mutations on the equivalent K58$^{\text{CyaA-CA}}$ and H298$^{\text{CyaA-switch-B}}$ in CyaA (Glaser *et al*, 1989; Glaser *et al*, 1991) or H577$^{\text{EF-switch-B}}$ in EF (Drum *et al.*, 2002) also largely reduce their CaM-stimulated AC activity. Our data show that the two residues are correctly positioned and stabilised with two metal ions only after cofactor and substrate

binding and LID/C_B closure (Fig. 6A, 7A, S7). Further investigation is required to determine whether H3753$^{VnE\text{-}switch\text{-}B}$ (or the equivalent histidine in other NC toxins) is critical for initiating 3'OH deprotonation via Mg^{2+}_A, and K3528$^{VnE\text{-}CA}$ (or equivalent) for the phosphoryl transfer step in a manner analogous to R1029$^{tmAC\text{-}IIC2}$ in tmAC. A key element in the ability of Vn-ExoY, and most likely other NC toxins, to catalyse the cyclisation reaction of nucleotides of different sizes is the tight coordination of the triphosphate moiety of purine and pyrimidine nucleotides after closure of their C_B/LID subdomain. In the structure of Vn-ExoY bound to actin and 3'dCTP-2Mg^{2+}, the hydrogen bond network around the ribose and base is weaker than for 3'dATP. This probably reduces the stability of pyrimidine nucleoside binding in the NBP of activated Vn-ExoY and may explain why Vn-ExoY remains less efficient as CC than AC. It will be interesting to compare these structural insights into the broad NC activity of NC toxins with the functional specificities of newly identified bacterial PycC pyrimidine cyclases, which appear to contain conserved class III cyclase catalytic elements (Tal *et al.*, 2021).

Despite the lack of tertiary and quaternary structural homology, class II (NC toxins) and class III ACs may share some important determinants of catalysis. Upon activation, the collapse of their different ATP/metal-ion coordination regions towards the active site brings together and constrains all interactions with the various parts of the nucleotide substrate and the metal ions. This open-closed transition of their catalytic domains may also facilitate the conformational changes of the substrate predicted by computational studies during the cyclisation reaction (Mones *et al.*, 2013; Steegborn, 2014; Hahn *et al.*, 2015; Jara & Martinez, 2016). For the catalytic domain of cofactor-activated NC toxins, the substrate-induced fit mechanism is controlled by the C_B/LID subdomain, which is near the cofactor-binding site. Its intrinsic flexibility and substrate-induced closure ensure both good substrate specificity and efficient enzymatic turnover through repeated closure and opening of the C_B/LID for substrate loading/processing and product release (Fig. 8). Without cofactor binding, substrate-induced NBP closure remains unproductive in catalysing the reaction because switch A and B, and thus part of the coordination of Mg^{2+}_A and the substrate nucleoside moiety (Fig. 6A, 7A), are not properly stabilised. Our data suggest that the substrates enter the active site of NC toxins by binding through their triphosphate moiety. Indeed, the base-coordinating switch A is initially unstable and incorrectly positioned in ExoY toxins. This is consistent with the ability of NC toxins to use nucleotide substrates with varying base sizes. Our study therefore provides a better understanding of how the NBP of NC toxins is regulated and gradually switches to a catalytic conformation. It opens up new structural perspectives for identifying ways to inhibit these NC toxins, which are involved in a wide range of bacterial infections.

Materials and Methods

The SI Appendix includes the protein constructs, design of the chimeras used for crystallization and methods for protein expression and purification. Methodologies for pyrene-actin polymerization assays, analytical ultracentrifugation, microscale thermophoresis experiments, NC activity assays, protein crystallization and crystallography are also detailed. Data collection experiments were performed on the PROXIMA-1 and PROXIMA-2 beamlines at synchrotron SOLEIL (Saint-Aubin, France).

Data Availability. X-ray crystallography data have been deposited in Protein Data Bank (8BJH, 8BJI, 8BJJ, 8BR1, 8BO1, 8BR0). See Table S1 in SI Appendix for further details.

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The authors declare no competing interest.

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Figure legends

Fig. 1. Vn-ExoY sequesters actin-ATP/ADP monomers in actin self-assembly by interacting with modest affinity. (A-C) Polymerisation time course of 4 µM ATP-Mg-G-actin (5 % pyrenyl-labelled, (A-B)) or 10 µM ADP-Mg-G-actin (10 % labelled, (C)) in the absence (black) and presence of Pa-ExoY^{K81M} (A) or Vn-ExoY^{DM} (B-C) at the indicated concentrations (µM). **(D)** Initial rates of barbed-end (dark blue) or pointed-end (light blue) growth from spectrin-actin or gelsolin-actin seeds, respectively, were measured with 1.5 µM G-actin (10 % pyrenyl-labelled) and increasing concentrations of Vn-ExoY^{DM}. Initial rates were normalised to the initial elongation rate with free G-actin from a linear fit to the first 120 s of each time course. Vn-ExoY^{DM} inhibits both barbed and pointed-end growth by sequestering G-actin with the indicated Kd (µM). **(E)** AUC-FDS measurement of the binding affinity of Vn-ExoY for G-actin. The sedimentation coefficient distribution c(s) (left panel) of Alexa-488-labelled-G-actin-ATP (60 nM) bound to the actin-polymerisation inhibitor Latrunculin A (2.5 µM) was determined in the absence and presence of increasing concentrations of unlabelled Trx-Vn-ExoY (from 0.5 to 50 µM). The resulting sw isotherms from c(s) integration are shown in the right panel. In the isotherm plots, the solid circles are the sw data from the dilution series. The solid line is the best-fit isotherm giving an estimated Kd of 11.7 µM for the interaction between Trx-Vn-ExoY and G-actin-ATP-Latrunculin-A.

Fig. 2. Vn-ExoY is activated by profilin-bound G-actin and inhibits spontaneous or regulated assembly of actin-profilin on F-actin barbed-ends *in vitro*. (A) cAMP synthesis by G-actin-activated Vn-ExoY in the presence of G-ABPs. Reactions containing 5 ng of Vn-ExoY and Latrunculin-A-bound actin at the indicated concentrations (µM) were initiated with 2 mM ATP and incubated at 30°C for 30 min. cAMP synthesis was measured in the absence (black) or presence of two high concentrations (10, 25 µM) of profilin (green), a sequestering β-thymosin (pink) or profilin-like WH2 (purple) domains. The Kd of their interaction with G-actin is 0.1, 0.5 and 0.5 µM, respectively (Husson *et al.*, 2011; Didry *et al.*, 2012). **(B)** Formation of the Vn-ExoY:actin:profilin complex by AUC-FDS. The c(s) distribution of Alexa-488-labelled-G-actin (60 nM) was determined alone (grey line) and in the presence of either profilin (8 µM; green line), Trx-Vn-ExoY (24 µM; yellow line) or both proteins (red line). **(C)** Measurement of Trx-Vn-ExoY^{DM} binding to free or profilin-bound actin from fluorescence changes in low ionic strength MST experiments. Titration of Alexa-488-labelled, Latrunculin-A-bound ATP-G-actin (0.075 µM) alone (cyan) or bound to 4 (red) and 7.9 (green) µM profilin by Trx-Vn-ExoY^{DM} for a 2-fold dilution series from 108 to 0.033 µM of Trx-Vn-ExoY^{DM} in MST-optimised buffer (see Methods). The inset shows the fluorescence changes in the raw data. The table shows the resulting Kd.

Error bars are s.d. (n≥3). **(D)** Vn-ExoY inhibits barbed-end elongation by actin-profilin. Initial barbed-end growth rates from spectrin-actin seeds (0.36 nM) were measured as in figure 1D using 2 µM G-actin (5 % pyrenyl-labelled) and increasing concentrations of profilin in the absence and presence of Vn-ExoY at the indicated concentrations. **(E-F)** Vn-ExoY inhibits formin- (E) or VASP-mediated (F) barbed-end elongation from actin-profilin. Barbed-end growth from spectrin-actin seeds (0.13 nM), 1 µM G-actin (5 % pyrenyl-labelled), and 8 µM profilin was measured in the absence or presence of 2.1 nM of capping protein (CP), 3.2 nM of FH1-FH2 domains of mDia1 formin (denoted by F) (E) or 0.22 µM of VASP (V) (F) at the indicated concentrations of Vn-ExoY (VnE).

Fig. 3. Crystal structures of Vn-ExoY in different conformational states bound to actin:profilin or actin, starting with a chimeric protein containing three fused functional domains including Vn-ExoY. (A) Domain diagrams of: (1) the VnE/VnE^{DM}-PRM-Prof chimera used to initiate the crystallographic study containing Vn-ExoY extended at the C-terminus (ExoY$^{3455-3896}$, numbering from Uniprot accession no. (AC) F0V1C5) fused to a short PRM/Proline-Rich-Motif capable of binding to profilin, and human profilin-1 (Uniprot AC P07737), and (2) an optimised shorter Vn-ExoY$^{3455-3863}$ construct that remains as active as our original construct Vn-ExoY$^{3412-3873}$ (Belyy *et al.*, 2016). **(B)** Synthesis of cAMP by actin-activated Vn-ExoY or VnE-PRM-Prof. VnE-PRM-Prof exhibits strong actin-dependent AC activity that saturates at much lower G-actin concentrations. Reactions containing 5 ng Vn-ExoY or 2 ng VnE-PRM-Prof and actin at the indicated concentrations were initiated with 2 mM ATP and incubated at 30°C for 10 or 30 min. **(C)** VnE^{DM}-PRM-Prof sequestered G-actin-ATP with 100-fold higher affinity than Vn-ExoY^{DM} (residues 3412-3873) in the barbed-end elongation. Initial barbed-end growth rates from spectrin-actin seeds were measured using 2 µM actin (3 % pyrenyl-labelled) and increasing concentrations of VnE^{DM}-PRM-Prof (in red) or Vn-ExoY^{DM} (in blue). **(D)** Structure of the nucleotide-free VnE^{DM}-PRM-Prof chimera bound to actin-ADP-LatB. In Vn-ExoY, the C_A subdomain and the flexible switch A, switch B, switch C and LID/C_B subdomain are shown in yellow, blue, cyan, purple and orange, respectively, here and in all subsequent figures. Disordered regions are shown as dotted lines, the position of the ATP substrate in Vn-ExoY NBP by its surface in red and the four actin subdomains by numbers in grey. Conserved interactions corresponding to hotspots are indicated by black labels, with the Vn-ExoY and actin side chains shown as green and red sticks, respectively. The sulphate ion bound in the VnE-PRM-Prof-SO_4^{2-}:actin-ADP structure is overlaid. **(E)** Structure of nucleotide-free Vn-ExoY in complex with actin-ATP-LatB and profilin (Vn-ExoY-SO_4^{2-}:actin-ATP-LatB:profilin). **(F)** Structure of 3'dATP-bound Vn-ExoY in complex with ATP-actin-LatB (Vn-ExoY-3'dATP-2Mg^{2+}:actin-ATP-LatB).

Fig. 4. Sequence diagram of the structural rearrangements in actin-bound nucleotide-free Vn-ExoY upon substrate binding. (A) Porcupine plot illustrating the direction of Cα atom movement (Schrödinger & DeLano, 2020) between the nucleotide-free and 3'dATP-bound Vn-ExoY conformations. Red arrow length proportional to Cα atom motion amplitude. **(B)** Vn-ExoY conformations in the Vn-ExoY-SO_4^{2-}:actin-ATP-LatB:profilin and Vn-ExoY-3'dATP-$2Mg^{2+}$:actin-ATP-LatB structures (actin and profilin omitted) overlaid on their C_A subdomain. Residues with significant solvent-accessible areas (BSAs) buried by actin are indicated by yellow (BSA ≤ 35 $Å^2$), green (between 35 and 70 $Å^2$) and pink (≥ 70 $Å^2$) spheres on Cα. **(C)** Vn-ExoY-buried actin residues detailing contacts between proteins. The four numbered actin subdomains are shown in different colours. Vn-ExoY residues are boxed with the same colour code as in (A,B). **(D)** The LID/C_B, switch A, B and C regions of Vn-ExoY are coloured red, light blue, grey and violet in Vn-ExoY-SO_4^{2-}:actin-ATP-LatB:profilin, and orange, blue, cyan and purple in Vn-ExoY-3'dATP-$2Mg^{2+}$:actin-ATP-LatB, respectively. D-1) In the nucleotide-free state, switch B and the beginning of switch C, which contacts switch A and B, are ordered but show relatively high B-factors in Vn-ExoY. The structures are drawn in a cartoon putty representation (Schrödinger & DeLano, 2020), where the backbone thickness is proportional to the B-factor value. The black dotted lines represent intramolecular interactions. D-2) The conformations of switch B and C, and residues (L3530-F3531^{VnE}) near the LID hinge region V3532-K3533^{VnE} are incompatible with 3'dATP binding or the final stabilised conformation of switch A induced by 3'dATP binding. To avoid steric clashes (red explosion symbols), they have to be rearranged. D-3) Structural rearrangement of switch B and LID. LID closure is stabilised by electrostatic bonds between K3535$^{VnE\text{-}LID}$ or R3559$^{VnE\text{-}LID}$ and D3766$^{VnE\text{-}switch\text{-}B}$ side chains. The red arrows indicate how the different regions move. D-4) Structural rearrangements of switch A and C. D-5) In this 3'dATP-bound state, switch B and C have B-factors of the same order as those in the overall structure, and the entire switch A stabilises on G-actin. Only the region of switch A (residues 3686-3719) that binds between actin subdomains 2 and 4 retains relatively high B-factors.

Fig. 5. Importance of the switch C at the actin:Vn-ExoY interface. (A) cAMP synthesis catalysed by wild-type or switch C mutants of Vn-ExoY (Vn-ExoY$^{3455\text{-}3863}$) activated by G-actin. Reactions containing 2.5 or 5 ng for Vn-ExoY and 5, 50 or 100 ng for Vn-ExoY mutants and 3 µM G-actin-ATP-LatB were started with 2 mM ATP and incubated at 30°C for 15 or 30 min. Values are averages of reactions with the indicated enzyme concentrations. **(B)** The amphipathic α-helix at the extreme C-terminus of Pa-ExoY (residues 365-376$^{PaE\text{-}switch\text{-}C}$) is shown in a helical wheel projection using the HeliQuest analysis (http://heliquest.ipmc.cnrs.fr/). **(C)** cGMP synthesis catalysed by wild-type Pa-ExoY or switch C mutants activated by F-actin. Reactions containing 5 ng Pa-ExoY^{wt} or Pa-ExoY(R364A,

K347A, or H352A) or 1 μg Pa-ExoY(F367A-L371A-F374A) and 3 μM F-actin were started with 2 mM GTP and incubated at 30°C for 10 min. Error bars in (A,C) correspond to the s.d. of at least two experimental replicates. In F-actin:Pa-ExoY structure (Belyy *et al.*, 2021), the K347, H352 and R364 side chains are at distances of 3.1, 4.5 and 4.1 Å from the $D25^{Actin}$ side chain, respectively. **(D)** Phylogenetic tree showing the relationships between ExoY-like proteins/effector domains produced by different β- and γ-proteobacteria. Vn- and Pa-ExoY are representative of the distant ExoYs. ExoYs are classified as Vn- or Pa-ExoY-like homologues based on both their sequence similarity and the predicted complex with actin by AlphaFold (Jumper *et al*, 2021) (see Fig. S10 for more details). A + sign indicates that the Vn- or Pa-ExoY residue/structural element is conserved at the same position in the ExoY-like homologue's sequence and predicted structure.

Fig. 6. Model of ATP-binding in activated Vn-ExoY and mammalian transmembrane AC (tmAC) and residues important for the purinylyl cyclase activities of Vn- and Pa-ExoY. (A) Model of ATP-binding in activated-Vn-ExoY based on its interactions with 3'dATP in the Vn-ExoY-3'dATP-2*Mg^{2+}:ATP-actin-LatB structure. Thresholds used for interaction detection are those of the PLIP (Adasme *et al*, 2021) and Arpeggio (Jubb *et al*, 2017) web servers and the PoseView tool in the ProteinsPlus web server (Schoning-Stierand *et al*, 2020). **(B)** The model-phased anomalous difference Fourier map from data collected at 6.55 keV, shown as a green mesh contoured at 5.0 σ, confirms the presence of two Mn^{2+} (grey spheres) bound to 3'dATP in Vn-ExoY NBP. **(C)** cAMP synthesis catalysed by wild-type or mutants of Vn-ExoY ($Vn\text{-}ExoY^{3455\text{-}3863}$) activated by G-actin. Reactions containing 10 ng Vn-ExoY, 100 ng Vn-ExoY(K3533Q) or 1 μg Vn-ExoY(K3528Q, K3535Q, or H3753Q) and 3 μM of LatB-Mg-G-actin were started with 2 mM ATP and incubated at 30°C for 10-30 min. **(D)** cGMP synthesis catalysed by wild-type or mutants of Pa-ExoY activated by F-actin. Reactions containing 10 ng $Pa\text{-}ExoY^{wt}$/Pa-ExoY(K86Q or K88Q) or 8 μg Pa-ExoY(K81Q or H291Q) and 3 μM of Mg-F-actin were started with 2 mM GTP and incubated at 30°C for 10-30 min. **(E)** The LID/C_B subdomain of EF occupies different positions relative to the C_A subdomain between the nucleotide-free (1xfz, 1xfy) (Shen *et al.*, 2005) and nucleotide-bound (1k90) (Drum *et al.*, 2002) structures of CaM-bound EF. It rotates between the 1xfz and 1k90 PDBs by 16° as a rigid body. The structures are superimposed on their C_A subdomains shown in yellow. **(F)** Model of ATP-binding in activated class III tmAC based on the structure of the type V AC C1a/type II AC C2 heterodimer bound to the ATP analogue RP-ATPαS (PDB 1CJK) (Tesmer *et al.*, 1999). Activated Vn-ExoY and tmAC closed NBPs have similar interactions with ATP analogues. For example, their conserved asparagine $N3759^{VnE\text{-}switch\text{-}B}$ or $N1025^{tmAC\text{-}IIC2}$ similarly hydrogen-bonds the ribose ring O4'-oxygen of ATP analogues. In tmAC, the interaction of $N1025^{tmAC\text{-}}$

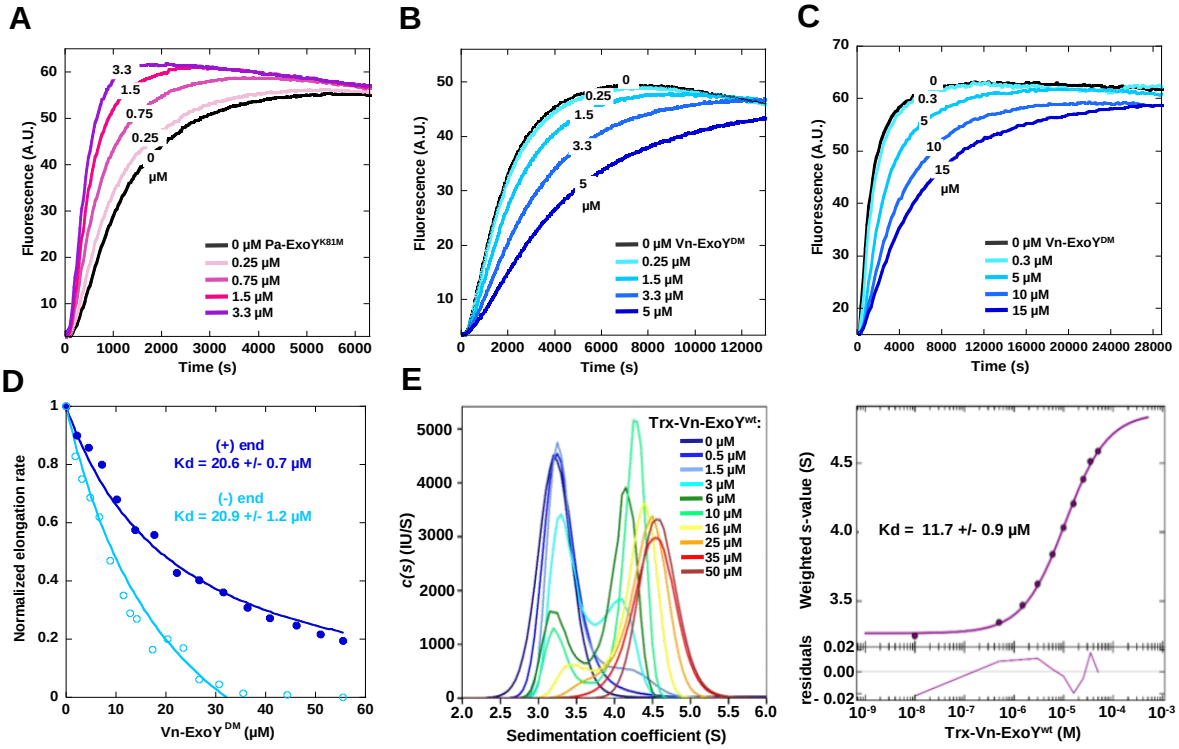
^{IIC2} with ATP is essential for correct ribose positioning during catalysis (Yan *et al.*, 1997; Tesmer *et al.*, 1999).

Fig. 7. Model of CTP-binding in actin-activated Vn-ExoY and residues important for the CC or UC activity of Vn- and Pa-ExoY. (A) Model of CTP-binding in activated-Vn-ExoY based on its interactions with 3'dCTP in the Vn-ExoY-3'dCTP-2*Mn^{2+}:ADP-actin structure. Interaction detection thresholds are as in Fig. 6A, F. **(B)** cCMP synthesis catalysed by wild-type or mutants of Vn-ExoY activated by G-actin. Reactions containing 100 ng Vn-ExoYwt or 1 µg-100 ng (mean) Vn-ExoY(K3528Q, K3533Q, K3535Q, or H3753Q) and 6 µM of Lat.B-Mg-G-actin were started with 2 mM CTP and incubated at 30° C for 60 min. **(C)** cCMP and cUMP synthesis catalysed by wild-type or mutants of Pa-ExoY activated by F-actin. cCMP synthesis was performed by using 1µg-100 ng (mean) Pa-ExoY^{wt} or Pa-ExoY(K81Q, K86Q, K88Q, or H291Q) and 6 µM of Mg-F-actin and reactions were started with 2 mM CTP and incubated at 30° C for 60 min. cUMP synthesis was performed by using 100-200 ng (mean) Pa-ExoY^{wt} or 1000-100 ng (mean) Pa-ExoY(K81Q, K86Q, K88Q, or H291Q) and 6 µM of Mg-F-actin and reactions were started with 2 mM UTP and incubated at 30° C for 60 min. In the absence of G-/F-actin, the basal CC and UC activity of Vn- and Pa-ExoY are 0.007 and 0.003 pmol/min/ng of the enzyme, respectively.

Fig. 8. Model of the activation mechanism and cytotoxic effects of Vn-ExoY elicited by its interaction with actin:profilin. (A) Once secreted by bacterial T1SS, the MARTX toxin binds to the target cell membrane, translocates and releases its various MARTX effector modules into host cells (Ziolo *et al.*, 2014), including Vn-ExoY. **(B)** In isolation, Vn-ExoY's three switch regions and CB/LID domain are flexible and unstable, and the enzyme is inactive. **(C)** The interaction of Vn-ExoY with the actin:profilin complex is first tightly docked to actin by switch C. This first step brings together and stabilises only part of the substrate and metal ion binding sites: it initiates stabilisation of switch B and brings switch A closer to the NBP. This intermediate activated conformation allows the active site to 'breathe', particularly at the C_B/LID subdomain of NC toxins, which is close to the cofactor binding site. The C_B/LID subdomain of NC toxins can therefore continue to adopt a variety of positions. **(D)** Substrate and metal ion binding promotes NC toxin C_B/LID closure. This allows the cofactor pre-stabilised active site of NC toxins to accommodate and ultimately constrain all substrate and metal ion interactions. By simply closing and opening the C_B/LID to load and process nucleotide substrates of different sizes (purine/pyrimidine nucleotides) and release the products, the large substrate-induced fit movement of the NC toxin C_B/LID subdomain ensures both good substrate specificity and efficient enzymatic turnover. **(E)** The formation of an abortive ternary complex, recruited by ABPs through their

PRMs (present in formin FH1) and sequestering G-actin:profilin, may act in two ways. It confines Vn-ExoY's toxic NC activity to sites of active actin remodelling. At these sites, G-actin:profilin and elongation-promoting factors, in close cooperation with cAMP-dependent signalling, fine-tune the homeostasis of F-actin networks (Howe, 2004; Yamashita *et al.*, 2011; Skruber *et al.*, 2020; Simao *et al.*, 2021). It may also locally interfere with the activity or regulation of various elongation factors, such as VASP, Arp2/3 or formin (shown here inducing membrane protrusions), if the proper functioning of their PRMs, which deliver G-actin to the barbed ends of F-actin, is impaired.

Figure 1


A
Fluorescence (A.U.)
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3.3
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0.25
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µM
0 µM Pa-ExoY^{K81M}
0.25 µM
0.75 µM
1.5 µM
3.3 µM
B
Fluorescence (A.U.)
Time (s)
0
0.25
1.5
3.3
5
µM
0 µM Vn-ExoY^{DM}
0.25 µM
1.5 µM
3.3 µM
5 µM
C
Fluorescence (A.U.)
Time (s)
0
0.3
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10
15
µM
0 µM Vn-ExoY^{DM}
0.3 µM
5 µM
10 µM
15 µM
D
Normalized elongation rate
Vn-ExoY^{DM} (µM)
(+) end
Kd = 20.6 +/- 0.7 µM
(-) end
Kd = 20.9 +/- 1.2 µM
E
c(s) (IU/S)
Sedimentation coefficient (S)
Trx-Vn-ExoY^{wt}:
0 µM
0.5 µM
1.5 µM
3 µM
6 µM
10 µM
16 µM
25 µM
35 µM
50 µM
Weighted s-value (S)
Kd = 11.7 +/- 0.9 µM
residuals
Trx-Vn-ExoY^{wt} (M)

Figure 2

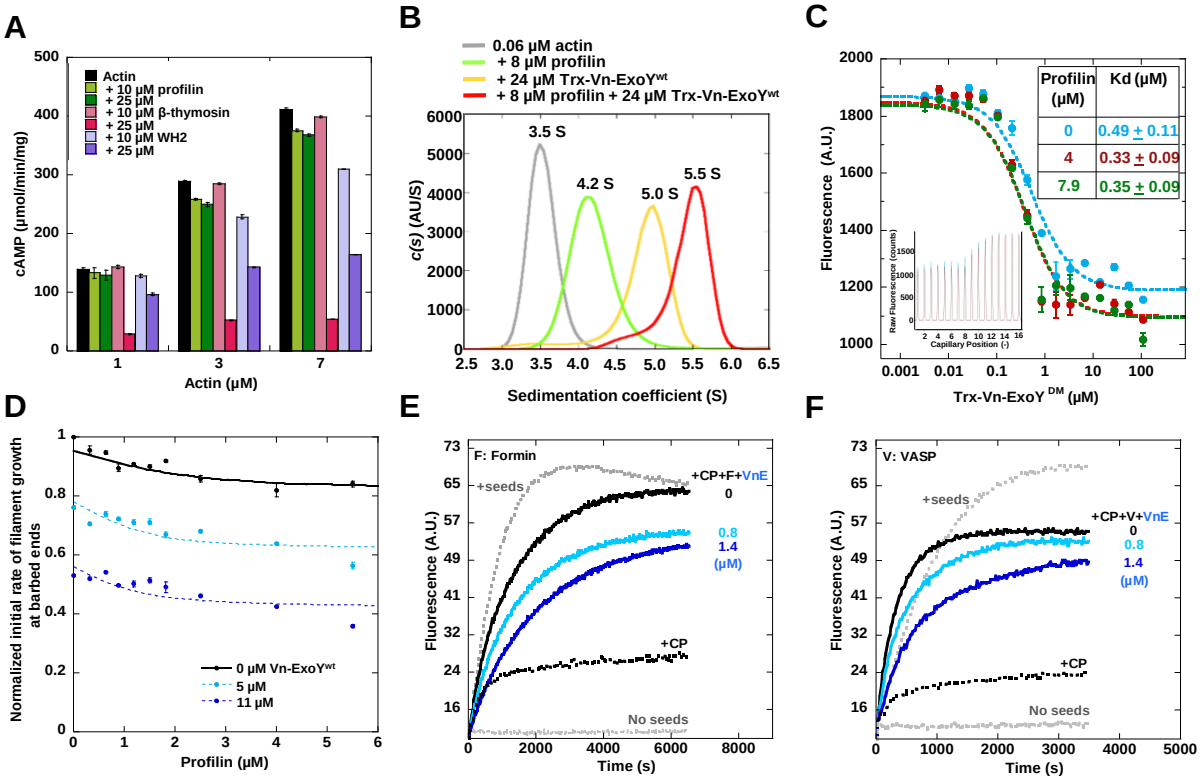

A
Actin
+ 10 µM profilin
+ 25 µM
+ 10 µM β-thymosin
+ 25 µM
+ 10 µM WH2
+ 25 µM
cAMP (µmol/min/mg)
Actin (µM)
B
0.06 µM actin
+ 8 µM profilin
+ 24 µM Trx-Vn-ExoY^wt
+ 8 µM profilin + 24 µM Trx-Vn-ExoY^wt
3.5 S
4.2 S
5.0 S
5.5 S
c(s) (AU/S)
Sedimentation coefficient (S)
C
Profilin (µM)
Kd (µM)
0
0.49 ± 0.11
4
0.33 ± 0.09
7.9
0.35 ± 0.09
Fluorescence (A.U.)
Trx-Vn-ExoY DM (µM)
Raw Fluorescence (counts)
Capillary Position (-)
D
Normalized initial rate of filament growth at barbed ends
0 µM Vn-ExoY^wt
5 µM
11 µM
Profilin (µM)
E
F: Formin
+seeds
+CP+F+VnE
0
0.8
1.4
(µM)
+CP
No seeds
Fluorescence (A.U.)
Time (s)
F
V: VASP
+seeds
+CP+V+VnE
0
0.8
1.4
(µM)
+CP
No seeds
Fluorescence (A.U.)
Time (s)

Figure 3

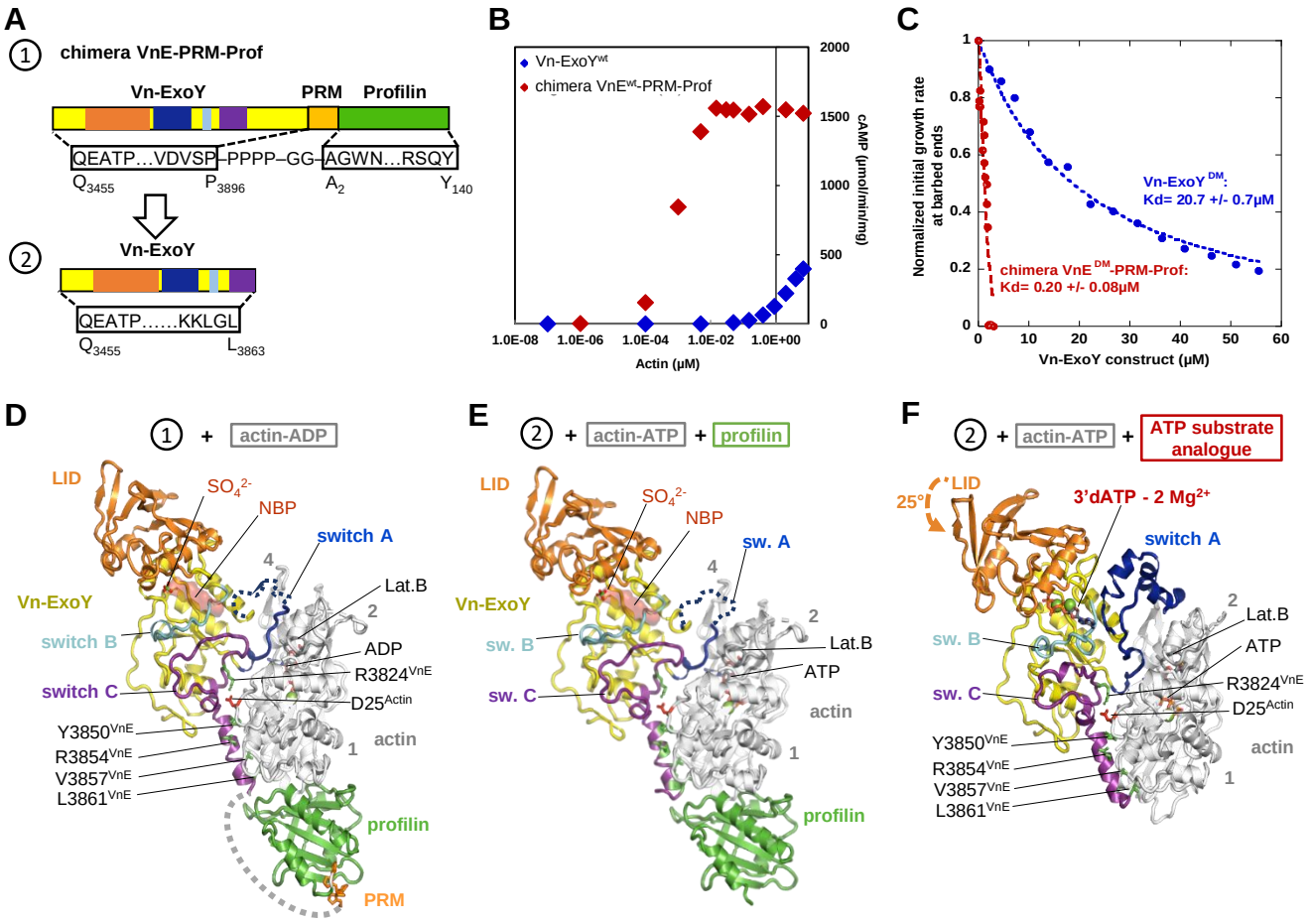

A
1 chimera VnE-PRM-Prof
Vn-ExoY
PRM
Profilin
QEATP…VDVSP–PPPP–GG–AGWN…RSQY
Q3455
P3896
A2
Y140
2
Vn-ExoY
QEATP……KKLGL
Q3455
L3863
B
Vn-ExoYwt
chimera VnEwt-PRM-Prof
cAMP (µmol/min/mg)
Actin (µM)
C
Normalized initial growth rate at barbed ends
Vn-ExoY construct (µM)
Vn-ExoY DM: Kd= 20.7 +/- 0.7µM
chimera VnE DM-PRM-Prof: Kd= 0.20 +/- 0.08µM
D
1 + actin-ADP
LID
SO4 2-
NBP
switch A
Lat.B
Vn-ExoY
switch B
ADP
R3824VnE
switch C
D25Actin
Y3850VnE
R3854VnE
V3857VnE
L3861VnE
actin
profilin
PRM
E
2 + actin-ATP + profilin
LID
SO4 2-
NBP
sw. A
Vn-ExoY
sw. B
Lat.B
ATP
sw. C
actin
profilin
F
2 + actin-ATP + ATP substrate analogue
25°
LID
3'dATP - 2 Mg2+
switch A
Lat.B
sw. B
ATP
R3824VnE
sw. C
D25Actin
Y3850VnE
R3854VnE
V3857VnE
L3861VnE
actin

Figure 4

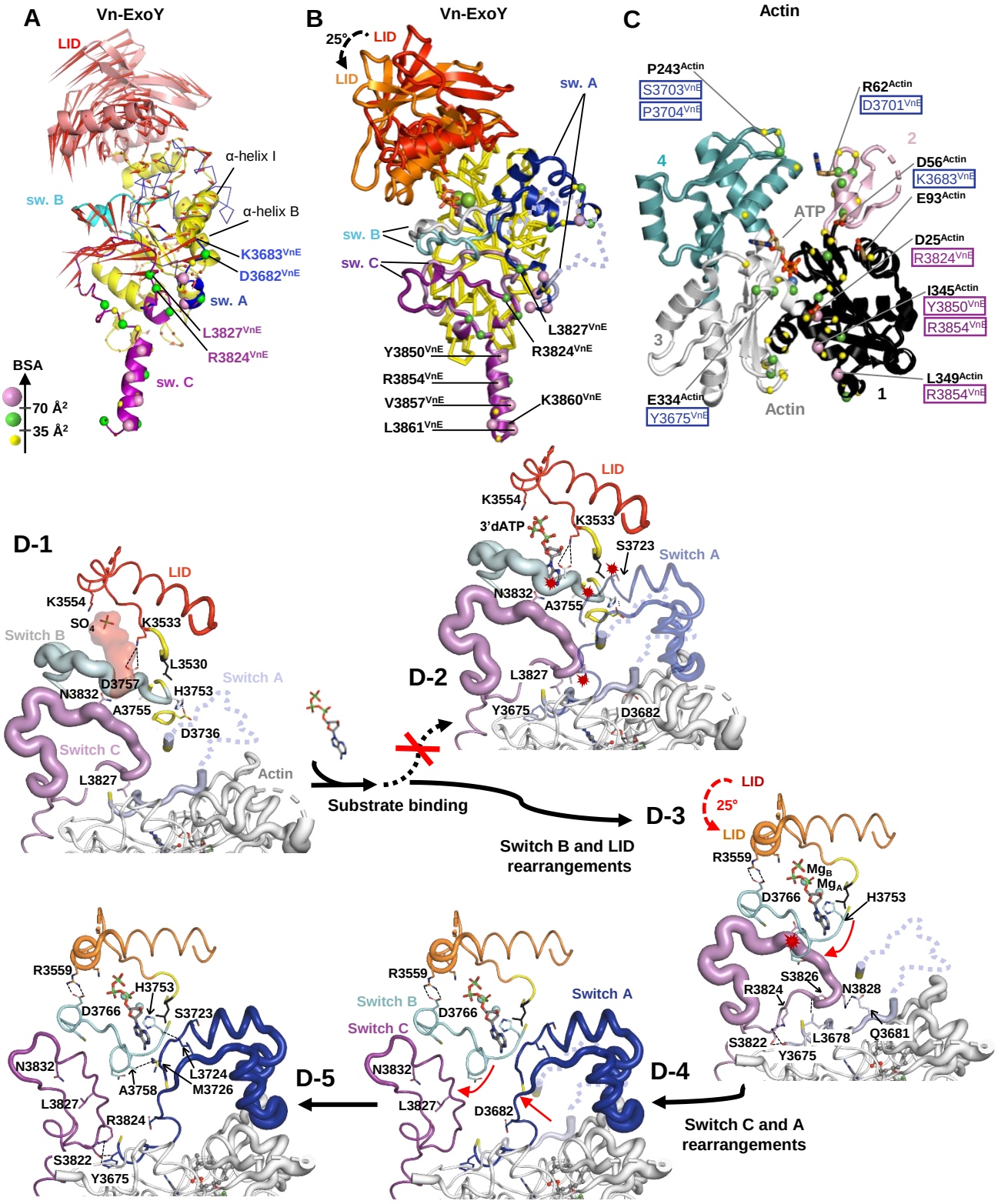

A
Vn-ExoY
LID
α-helix I
sw. B
α-helix B
K3683VnE
D3682VnE
sw. A
L3827VnE
R3824VnE
sw. C
BSA
70 Å²
35 Å²
B
Vn-ExoY
25°
LID
LID
sw. A
sw. B
sw. C
L3827VnE
R3824VnE
Y3850VnE
R3854VnE
V3857VnE
L3861VnE
K3860VnE
C
Actin
P243Actin
S3703VnE
P3704VnE
R62Actin
D3701VnE
2
4
D56Actin
K3683VnE
E93Actin
ATP
D25Actin
R3824VnE
I345Actin
Y3850VnE
R3854VnE
3
L349Actin
R3854VnE
1
E334Actin
Y3675VnE
Actin
D-1
LID
K3554
SO4
K3533
Switch B
L3530
Switch A
D3757
H3753
N3832
A3755
D3736
Switch C
L3827
Actin
Substrate binding
D-2
LID
K3554
3'dATP
K3533
S3723
Switch A
N3832
A3755
L3827
Y3675
D3682
Switch B and LID rearrangements
D-3
LID
25°
LID
R3559
MgB
MgA
D3766
H3753
S3826
R3824
N3828
S3822
L3678
Q3681
Y3675
Switch C and A rearrangements
D-4
R3559
Switch B
Switch A
D3766
Switch C
N3832
L3827
D3682
D-5
R3559
H3753
D3766
S3723
N3832
L3724
A3758
M3726
L3827
R3824
S3822
Y3675

Figure 5

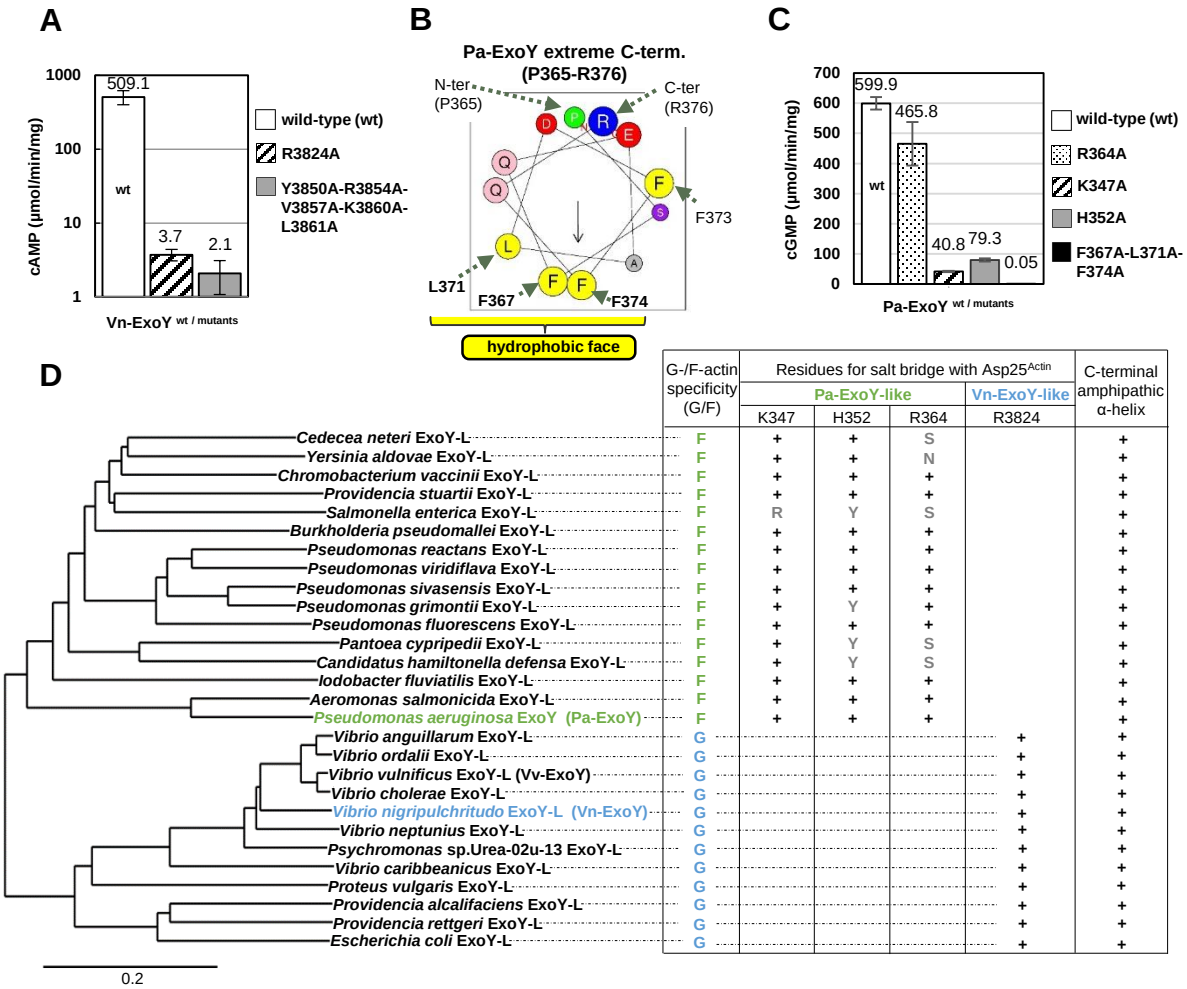
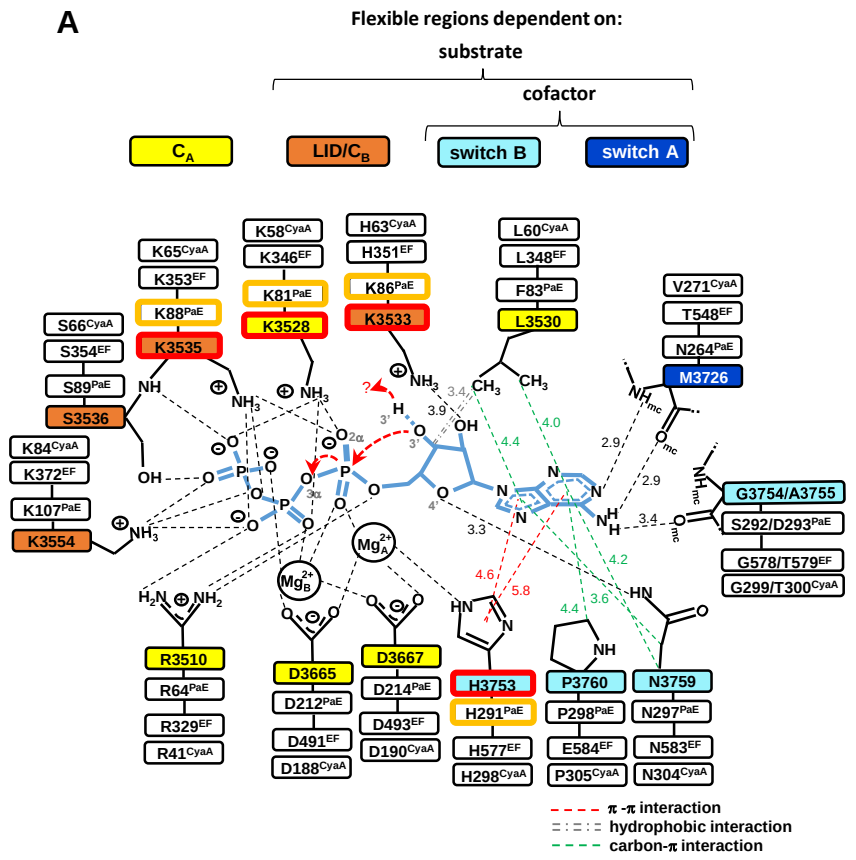
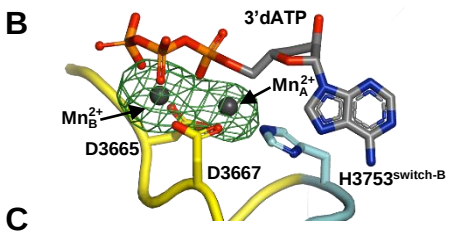
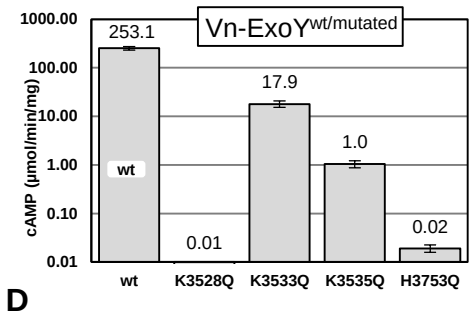
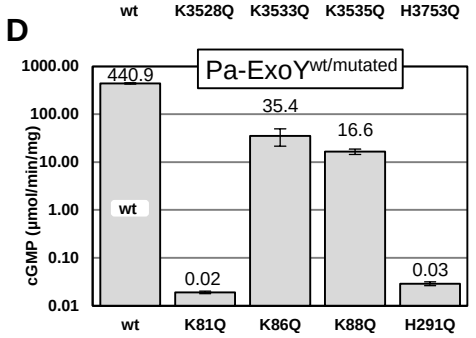

A
1000
100
10
1
cAMP (µmol/min/mg)
509.1
3.7
2.1
wt
wild-type (wt)
R3824A
Y3850A-R3854A-V3857A-K3860A-L3861A
Vn-ExoY wt / mutants
B
Pa-ExoY extreme C-term.
(P365-R376)
N-ter
(P365)
C-ter
(R376)
D
P
R
E
Q
Q
F
S
L
A
F
F
F373
L371
F367
F374
hydrophobic face
C
700
600
500
400
300
200
100
0
cGMP (µmol/min/mg)
599.9
465.8
40.8
79.3
0.05
wt
wild-type (wt)
R364A
K347A
H352A
F367A-L371A-F374A
Pa-ExoY wt / mutants
D
G-/F-actin specificity (G/F)
Residues for salt bridge with Asp25Actin
Pa-ExoY-like
Vn-ExoY-like
K347
H352
R364
R3824
C-terminal amphipathic α-helix
Cedecea neteri ExoY-L F + + S +
Yersinia aldovae ExoY-L F + + N +
Chromobacterium vaccinii ExoY-L F + + + +
Providencia stuartii ExoY-L F + + + +
Salmonella enterica ExoY-L F R Y S +
Burkholderia pseudomallei ExoY-L F + + + +
Pseudomonas reactans ExoY-L F + + + +
Pseudomonas viridiflava ExoY-L F + + + +
Pseudomonas sivasensis ExoY-L F + + + +
Pseudomonas grimontii ExoY-L F + Y + +
Pseudomonas fluorescens ExoY-L F + + + +
Pantoea cypripedii ExoY-L F + Y S +
Candidatus hamiltonella defensa ExoY-L F + Y S +
Iodobacter fluviatilis ExoY-L F + + + +
Aeromonas salmonicida ExoY-L F + + + +
Pseudomonas aeruginosa ExoY (Pa-ExoY) F + + + +
Vibrio anguillarum ExoY-L G + +
Vibrio ordalii ExoY-L G + +
Vibrio vulnificus ExoY-L (Vv-ExoY) G + +
Vibrio cholerae ExoY-L G + +
Vibrio nigripulchritudo ExoY-L (Vn-ExoY) G + +
Vibrio neptunius ExoY-L G + +
Psychromonas sp.Urea-02u-13 ExoY-L G + +
Vibrio caribbeanicus ExoY-L G + +
Proteus vulgaris ExoY-L G + +
Providencia alcalifaciens ExoY-L G + +
Providencia rettgeri ExoY-L G + +
Escherichia coli ExoY-L G + +
0.2

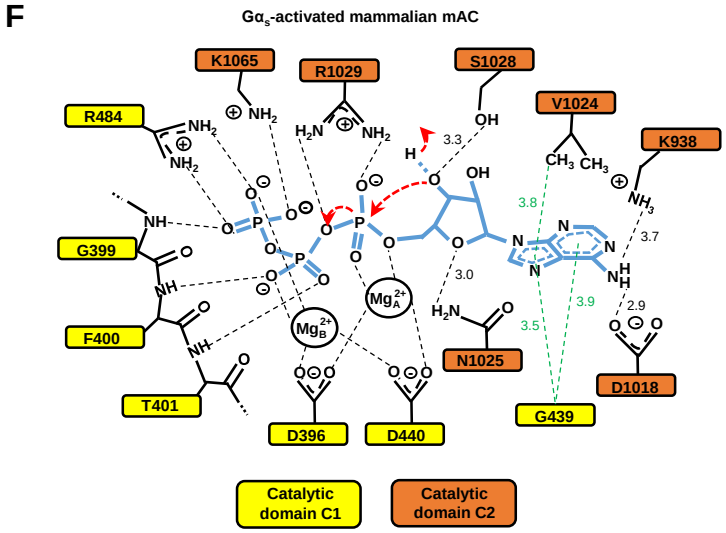
Figure 6


A
Flexible regions dependent on:
substrate
cofactor
C_A
LID/C_B
switch B
switch A
K65CyaA
K353EF
K88PaE
K3535
S66CyaA
S354EF
S89PaE
S3536
K84CyaA
K372EF
K107PaE
K3554
K58CyaA
K346EF
K81PaE
K3528
H63CyaA
H351EF
K86PaE
K3533
L60CyaA
L348EF
F83PaE
L3530
V271CyaA
T548EF
N264PaE
M3726
G3754/A3755
S292/D293PaE
G578/T579EF
G299/T300CyaA
R3510
R64PaE
R329EF
R41CyaA
D3665
D212PaE
D491EF
D188CyaA
D3667
D214PaE
D493EF
D190CyaA
H3753
H291PaE
H577EF
H298CyaA
P3760
P298PaE
E584EF
P305CyaA
N3759
N297PaE
N583EF
N304CyaA
π -π interaction
hydrophobic interaction
carbon-π interaction


B
3'dATP
Mn_A^2+
Mn_B^2+
D3665
D3667
H3753switch-B


C
Vn-ExoYwt/mutated
cAMP (µmol/min/mg)
1000.00
100.00
10.00
1.00
0.10
0.01
253.1
17.9
1.0
0.01
0.02
wt
wt
K3528Q
K3533Q
K3535Q
H3753Q


D
Pa-ExoYwt/mutated
cGMP (µmol/min/mg)
1000.00
100.00
10.00
1.00
0.10
0.01
440.9
35.4
16.6
0.02
0.03
wt
wt
K81Q
K86Q
K88Q
H291Q


F
Gα_s-activated mammalian mAC
K1065
R1029
S1028
V1024
K938
R484
G399
F400
T401
D396
D440
N1025
G439
D1018
Catalytic domain C1
Catalytic domain C2

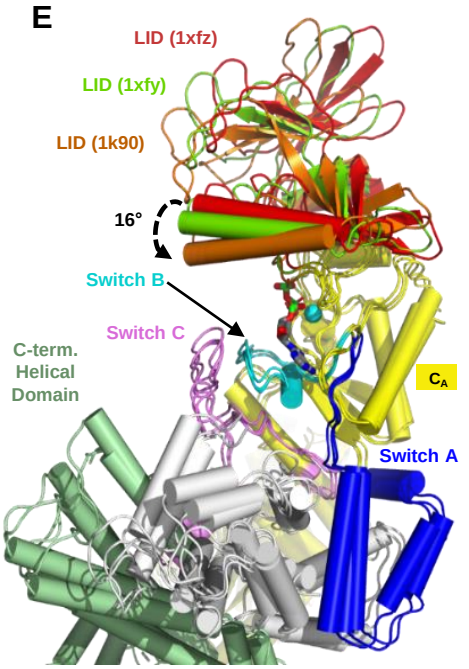

E
LID (1xfz)
LID (1xfy)
LID (1k90)
16°
Switch B
Switch C
C-term. Helical Domain
C_A
Switch A

Figure 7

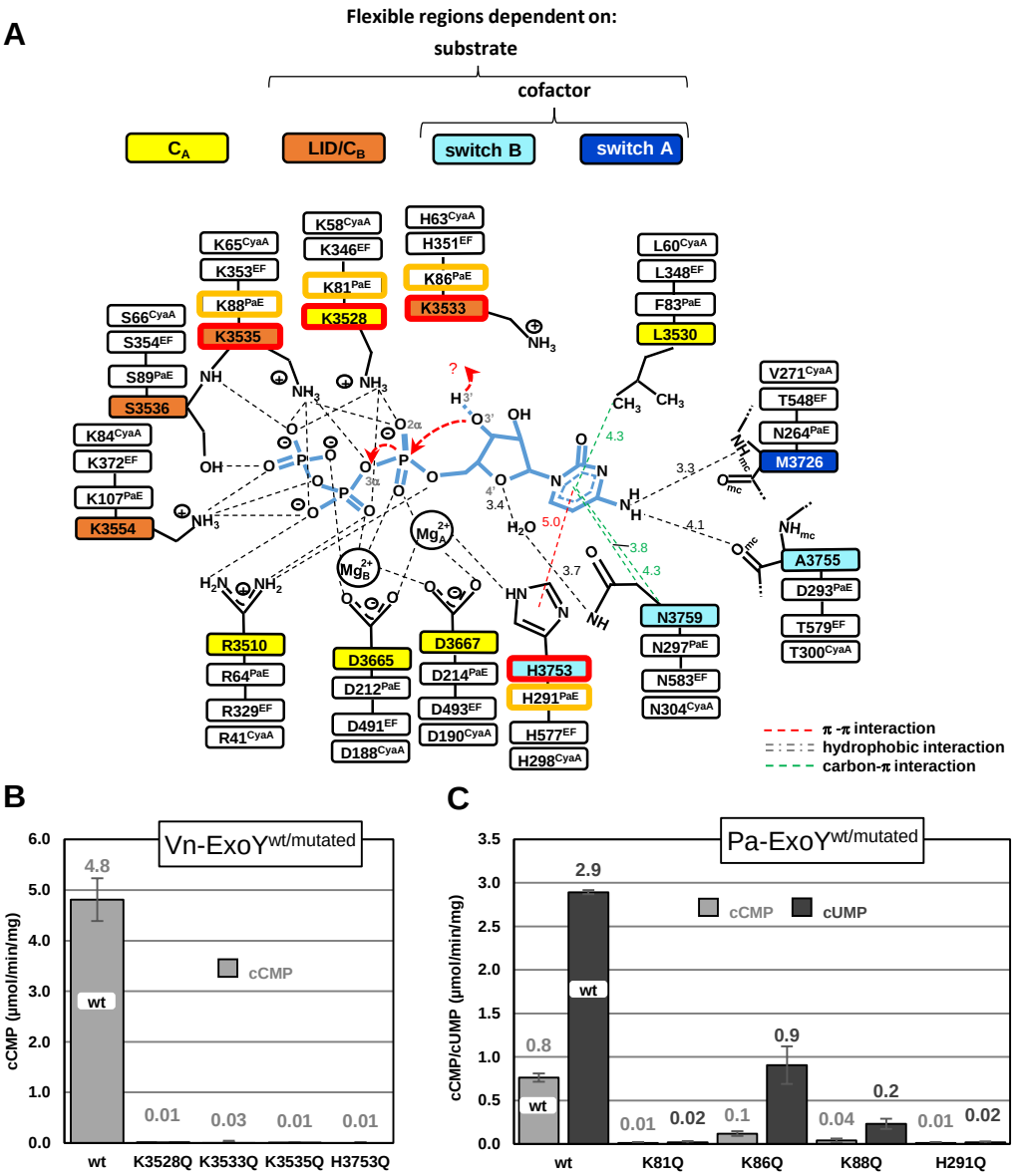
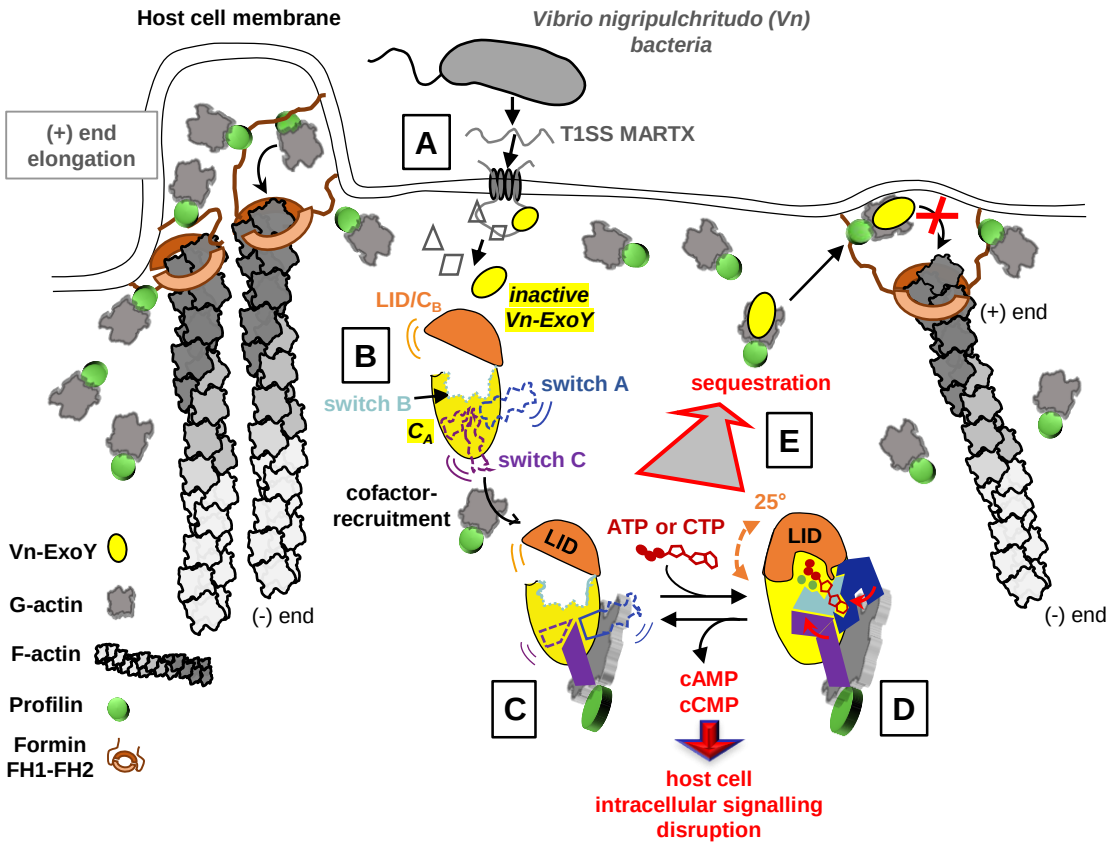

A
Flexible regions dependent on:
substrate
cofactor
C_A
LID/C_B
switch B
switch A
K65CyaA
K353EF
K88PaE
K3535
S66CyaA
S354EF
S89PaE
S3536
K84CyaA
K372EF
K107PaE
K3554
K58CyaA
K346EF
K81PaE
K3528
H63CyaA
H351EF
K86PaE
K3533
L60CyaA
L348EF
F83PaE
L3530
V271CyaA
T548EF
N264PaE
M3726
A3755
D293PaE
T579EF
T300CyaA
R3510
R64PaE
R329EF
R41CyaA
D3665
D212PaE
D491EF
D188CyaA
D3667
D214PaE
D493EF
D190CyaA
H3753
H291PaE
H577EF
H298CyaA
N3759
N297PaE
N583EF
N304CyaA
π -π interaction
hydrophobic interaction
carbon-π interaction
B
Vn-ExoY wt/mutated
cCMP (µmol/min/mg)
cCMP
wt
4.8
0.01
0.03
0.01
0.01
wt
K3528Q
K3533Q
K3535Q
H3753Q
C
Pa-ExoY wt/mutated
cCMP/cUMP (µmol/min/mg)
cCMP
cUMP
2.9
0.8
0.01
0.02
0.1
0.9
0.04
0.2
0.01
0.02
wt
K81Q
K86Q
K88Q
H291Q

Figure 8


Host cell membrane
Vibrio nigripulchritudo (Vn) bacteria
(+) end elongation
A
T1SS MARTX
LID/C_B
inactive Vn-ExoY
B
switch A
switch B
C_A
switch C
cofactor-recruitment
sequestration
E
25°
ATP or CTP
LID
LID
C
D
cAMP
cCMP
host cell intracellular signalling disruption
(+) end
(-) end
(-) end
Vn-ExoY
G-actin
F-actin
Profilin
Formin FH1-FH2