Pseudomonas aeruginosa exoenzyme Y directly bundles actin filaments

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Pseudomonas aeruginosa uses a type III secretion system (T3SS) to inject cytotoxic effector proteins into host cells. The promiscuous nucleotidyl cyclase, exoenzyme Y (ExoY), is one of the most common effectors found in clinical P. aeruginosa isolates. Recent studies have revealed that the nucleotidyl cyclase activity of ExoY is stimulated by actin filaments (F-actin) and that ExoY alters actin cytoskeleton dynamics in vitro, via an unknown mechanism. The actin cytoskeleton plays an important role in numerous key biological processes and is targeted by many pathogens to gain competitive advantages. We utilized total internal reflection fluorescence microscopy, bulk actin assays, and EM to investigate how ExoY impacts actin dynamics. We found that ExoY can directly bundle actin filaments with high affinity, comparable with eukaryotic F-actin–bundling proteins, such as fimbrin. Of note, ExoY enzymatic activity was not required for F-actin bundling. Bundling is known to require multiple actin-binding sites, yet small-angle X-ray scattering experiments revealed that ExoY is a monomer in solution, and previous data suggested that ExoY possesses only one actin-binding site. We therefore hypothesized that ExoY oligomerizes in response to F-actin binding and have used the ExoY structure to construct a dimmer-based structural model for the ExoY–F-actin complex. Subsequent mutational analyses suggested that the ExoY oligomerization interface plays a crucial role in mediating F-actin bundling. Our results indicate that ExoY represents a new class of actin-binding proteins that modulate the actin cytoskeleton both directly, via F-actin bundling, and indirectly, via actin-activated nucleotidyl cyclase activity.

The Gram-negative opportunistic human pathogen, Pseudomonas aeruginosa, is one of the most common causes of hospital-acquired infections. This bacterium utilizes a broad array of virulence factors to infect a wide range of tissues, including the skin, eyes, heart, and lungs. One of the most prominent virulence factors expressed during P. aeruginosa infection is the type III secretion system (T3SS), which functions as a molecular syringe and mediates the injection of proteinaceous effector toxins directly into the cytoplasm of host cells (1, 2). P. aeruginosa is known to secrete four effectors (ExoS, ExoU, ExoT, and ExoY), although typically no strain expresses more than three effectors. ExoS and ExoU appear to be nearly mutually exclusive, whereas ExoY and ExoT are encoded by nearly all pathogenic strains (3). ExoS and ExoT both exhibit G-protein activating and ADP-ribosylation activities, whereas ExoU is a potent phospholipase (4). ExoY is a promiscuous nucleotidyl cyclase that has recently been demonstrated to be activated by binding to actin filaments (F-actin), but not globular actin (G-actin) (5–8).

Actin is one of the most abundant proteins in the human body and forms the core of the eukaryotic cytoskeleton. Within the cell, actin exists in a state of dynamic equilibrium between monomeric G-actin and polymeric F-actin. This equilibrium is modulated by the activities of hundreds of actin-binding proteins (ABPs), which mediate key cellular functions, including division, motility, and phagocytosis (9–11). These ABPs are controlled by complex regulatory pathways, often mediated by GTPases, including Cdc42, Rho, and Rac (12). ABPs display a wide range of biological activities within the cell. Some ABPs alter the kinetics of actin polymerization, whereas others, such as fascin and fimbrin (human plastin homolog) reorganize actin filaments into cable-like bundles (13–15). The binding of ABPs alters the physical and dynamic properties of actin filaments and/or bundles. It has been demonstrated that these altered properties correlate with distinct physiological functions within the cell, such as motility and endocytosis (15, 16).

In addition to its regulation by Rho GTPases, some proteins that influence actin cytoskeletal dynamics are controlled by the second messenger cAMP, which exerts its regulatory effect upon actin via the cAMP-dependent protein kinase (PKA) and exchange protein activated by cAMP (EPAC) pathways (17, 18). cAMP is a ubiquitous signaling molecule synthesized from ATP by adenyl cyclases (19). Other cyclic nucleotides, including cUMP, cGMP, and cCMP, are also utilized as signaling molecules, yet cAMP appears to be the dominant signaling molecule (20). As a result, many bacteria have evolved virulence factors...

2 The abbreviations used are: T3SS, type III secretion system; ABP, actin-binding protein; EF, edema factor; TIRFM, total internal reflection fluorescence microscopy; SEC, size-exclusion chromatography; SAXS, small-angle X-ray scattering; PDB, Protein Data Bank; PISA, protein interface surface analysis; PMSF, phenylmethylsulfonyl fluoride.
that use adenylyl cyclase activity to interfere with host cytoskeleton regulatory processes to the benefit of the pathogen, such as Bordetella pertussis (CyaA), Bacillus anthracis (edema factor (EF)), and P. aeruginosa (ExoY) (21–23). Among these nucleotidyl cyclase toxins, ExoY is uniquely capable of influencing actin dynamics both directly, by binding to F-actin, and indirectly, via cyclase activity.

Although it has been shown that ExoY mediates cytoskeletal dysfunction in vivo, the exact cellular consequences vary by cell type, including generalized cytoskeletal breakdown, cell rounding, and membrane blebbing (7, 24–26). The enzymatic activity of ExoY has long been considered the primary mediator of these phenotypes. As a result, most studies of ExoY have focused on understanding the role of its enzymatic activity during infection. Recently, these efforts have culminated in the discovery that the enzymatic activity of ExoY is stimulated by binding to F-actin, but not G-actin (6). Furthermore, ExoY can alter actin dynamics in vitro. However, the mechanisms explaining how ExoY affects actin dynamics and how F-actin activates ExoY remain elusive. ExoY bears no significant sequence or structural homology to any characterized ABP (27). Thus, ExoY represents a new class of ABP. In this study, we utilized total internal reflection fluorescence microscopy (TIRFm) and bulk actin assays to characterize the fundamental properties of ExoY as an ABP. We have discovered that ExoY is able to directly bundle actin filaments, independent of its enzymatic activity. We hypothesize that this behavior is mediated by ExoY dimerization and present a structural model for F-actin bundling that is supported by biochemical and mutational studies.

Results

ExoY stimulates bundling of actin filaments in vitro

Actin filaments are complex dynamic macromolecules that result in heterogenic populations. TIRFm has emerged as one of the most informative techniques to investigate F-actin dynamics and organization in vitro (28, 29). Using TIRFm, we assessed the impact of ExoY on the organization of actin filaments under polymerizing conditions that promote the assembly of G-actin into F-actin. The F-actin nucleation and elongation rates were not noticeably altered in the presence of ExoY. However, we observed that ExoY facilitates the association of actin filaments along their length to form bundles of two (Fig. 1A and Movie S1) or more (Movie S2) filaments. An F-actin bundle is defined as a complex formed between two or more actin filaments, tethered lengthwise to each other by an accessory protein, and the bundling activity of ExoY is very similar to traditional cellular F-actin–bundling proteins that we have studied by TIRFm under similar conditions (15, 30, 31). Analysis of TIRFm time-lapse videos allowed for identification of the fast (barbed) and slow (pointed) growing ends of F-actin. Thus, we were able to determine that ExoY-stimulated F-actin bundles are composed of filaments with mixed polarity (Fig. S1 and Movie S3). Mixed-polarity bundles are commonly generated by other cellular F-actin–bundling proteins, such as α-actinin and fimbrin (15, 30, 31).

To further investigate whether ExoY promotes F-actin bundling, we then examined the ultrastructure of ExoY-mediated F-actin bundles using negative-stain EM. As predicted, in the absence of ExoY, only single actin filaments were observed, whereas bundles of F-actin were observed in the presence of ExoY (Fig. 1B). The ExoY-induced bundles were formed as three-dimensional cable-like clusters of actin filaments and appeared highly heterogeneous with great variation in the number of filaments within each bundle, consistent with the TIRFm. ExoY induced the formation of very tight F-actin bundles, to the point that we were unable to reliably measure the interfilament distance from the negatively stained micrographs, but we estimate the distance to be no more than several nanometers.

ExoY promotes F-actin bundling in a concentration-dependent manner. Using TIRFm, we found that ExoY effectively stimulated F-actin bundling at concentrations ≥200 nm. At concentrations of 100 and 150 nm, ExoY was able to induce transient bundle formation, where two actin filaments would briefly interact and then separate. Occasionally at these concentrations, two actin filaments would interact to form a stable bundle. No F-actin bundling was observed at ExoY concentrations <100 nm. These results indicate that ExoY is able to stimulate F-actin bundle formation with similar efficiency as eukaryotic F-actin–bundling proteins, such as fascin and α-actinin (15).

Enzymatic activity is not required for ExoY-mediated F-actin bundling

ExoY is known to function as a promiscuous nucleotidyl cyclase, and, to date, this enzymatic activity has been considered its dominant biological activity (7, 8). Therefore, we sought to determine whether ExoY enzymatic activity was required for
ExoY induces F-actin bundling

Figure 2. ExoY enzymatic activity is not required for actin bundling. A, frame-by-frame time lapse of two actin filaments bundling in the presence of catalytically inactive ExoYK81M. Actin (1.5 μm) is fluorescently labeled with Alexa Fluor 488. Images were captured via TIRFM. See Movie S1 for the full movie. Scale bars, 5 μm. B, negatively stained electron micrographs showing that actin filaments bundle in the presence of ExoYK81M (right). Actin filaments in the absence (left) and presence (center) of WT ExoY (panels from Video 18) are shown for comparison. Images were taken at ×11,000 magnification; scale bars, 200 nm. C, micrograph of the WT ExoY–actin bundle in vitreous ice at ×50,000 magnification; scale bar, 50 nm. D, micrograph showing a sample of actin filaments in the presence of catalytically inactive ExoYK81M in vitreous ice. The bundles formed by ExoYK81M do not survive the vitrification process. Magnification was ×50,000; scale bar, 50 nm.

The formation of F-actin bundles. Previous work has established that a single point mutation, K81M, renders ExoY catalytically inactive (7). We generated this mutation and purified the resulting construct, ExoYK81M. This construct was tested for its ability to stimulate F-actin bundling by TIRFM. ExoYK81M was found to promote the formation of bundles containing two or more actin filaments with similar efficiency compared with WT (Fig. 2A and Movie S1). We then confirmed ExoYK81M-mediated F-actin bundling via negative-stain EM (Fig. 2B). Interestingly, the bundles formed in the presence of WT ExoY versus ExoYK81M appeared to display distinctly different morphologies. The bundles formed in the presence of WT ExoY appeared very thick and three-dimensional, similar to a cable, whereas the bundles formed in the presence of ExoYK81M appeared much flatter, and easier to image, somewhat reminiscent of a ribbon.

We employed cryo-EM to investigate the apparent physiological differences with higher resolution. The F-actin bundles formed in the presence of WT ExoY appeared as they did in the negative-stain EM: thick cable-like structures with a high degree of heterogeneity (Fig. 2C). In contrast to the bundles formed in the presence of WT ExoY, the F-actin bundles formed in the presence of ExoYK81M fell apart during the grid-making process, and only single actin filaments were observed in the vitreous ice (Fig. 2D). This observation suggests that the visual differences between the F-actin bundles formed in the presence of WT versus inactive ExoY may correlate with distinct physical properties.

Bound ExoY tethers actin filaments within the bundle

We hypothesized that ExoY functions as a traditional F-actin–bundling protein, which physically binds multiple actin filaments simultaneously and organizes them into a bundle. To test this hypothesis, we utilized bulk F-actin sedimentation assays to biochemically characterize ExoY-actin filament binding and bundling. As ExoY and actin have molecular masses of roughly 42 kDa and are indistinguishable via SDS-PAGE, we fused a HaloTag (32) to the N terminus of ExoY, via a flexible linker, to generate a roughly 77-kDa ExoY fusion protein that allowed visual differentiation by SDS-PAGE. Analysis using the TIRFM polymerization assay revealed no difference in the bundling efficiency between WT ExoY and HaloTag-ExoY (data not shown). We then performed a high-speed sedimentation assay to characterize ExoY binding to preformed actin filaments (Fig. 3A). The SDS-PAGE revealed that HaloTagged ExoY readily sedimented with the actin filaments and that the interaction between ExoY and actin is not mediated by covalent interaction, unlike some bacterial toxins that are known to covalently cross-link actin molecules (33). The half-maximal effective concentration (EC50) for ExoY binding to F-actin was estimated to be 1.44 μM. The data were fit to the Hill equation, and the resulting Hill coefficient (n = 1.37) suggests a modest degree of cooperativity for ExoY binding to F-actin.

We then performed low-speed centrifugation F-actin sedimentation assays to assess the bundling efficiency of ExoY. ExoY appears to efficiently bundle F-actin, as the data from low-speed centrifugation experiments, once again fit to the Hill equation (n = 5.6), revealed an apparent EC50 for bundling of ~334 nM (Fig. 3B). This value fits well with the earlier observation by TIRFM that F-actin bundling is rare at ExoY concentrations below 200 nM, and no bundling was observed at concentrations below 100 nM. F-actin bundling is known to be a cooperative behavior, and the high Hill coefficient (n = 5.6) is similar to those observed for other cellular bundling proteins, including fascin and α-actinin (15). It must be noted that these calculations are based on the concentration of G-actin, rather than F-actin, which cannot be determined based on the stochastic nature of actin polymerization, meaning the actual Kd for ExoY–F-actin binding is significantly lower, likely in the low...
to mid nanomolar range. Direct comparison between the binding curves of ExoY and fission yeast fimbrin (30) reveals similar affinities, indicating that ExoY possesses a physiologically relevant affinity for F-actin comparable with eukaryotic ABPs.

The fact that HaloTagged ExoY readily sediments with F-actin bundles during the low-speed sedimentation assays provided strong evidence that ExoY functions as a traditional bundling protein. However, the possibility remained that this behavior was somehow an artifact caused by the addition of the HaloTag. To confirm that ExoY was present within the F-actin bundles, WT ExoY was labeled with Alexa Fluor 555 (ExoY 555), and two-color TIRFm was performed to visualize the association of ExoY with F-actin. We found that ExoY 555 decorated along F-actin nearly uniformly and was present within the bundles of F-actin (Fig. 3C and Movie S4). Together, we conclude that ExoY bundles F-actin via direct, physical interactions that do not require its nucleotidyl cyclase activity.

**Generation of a structural model explaining ExoY bundling**

There are two general mechanisms for how cellular actin-binding proteins promote F-actin bundling or cross-linking (15). The first type of bundling protein (e.g., fimbrin or fascin) has multiple F-actin–binding sites and acts as a monomer. The second type of bundling protein, exemplified by α-actinin and filamin, has only one F-actin–binding site per monomer and therefore must dimerize to tether actin filaments together. To assess the mechanism by which ExoY promotes F-actin bundling, we first determined the hydrodynamic properties of ExoY in solution. Based on size-exclusion chromatography (SEC), ExoY behaved as a monomer in solution (27). Due to the intrinsic issues of SEC-based determination of protein oligomeric state, we examined the oligomeric state of ExoY in solution using SEC-coupled small-angle X-ray scattering (SEC-SAXS) and compared the resulting data with a theoretical scattering profile calculated from the
previously published ExoY crystal structure (PDB code 5XNW) (Fig. 4) (27) to directly test whether ExoY indeed exists as a monomer in solution. Using the corrected Porod volume method, our SEC-SAXS data indicated that ExoY in solution is 42 kDa in size, nearly identical to the predicted molecular mass of an ExoY monomer (34). The SAXS profile also revealed that ExoY has a radius of gyration ($R_g$) of 25.3 Å and $D_{max} \approx 83.5 \pm 5.5$ Å, which is slightly larger than the calculated $R_g$ and $D_{max}$ ($\sim 23$ and $\sim 70$ Å, respectively) from the crystal structure of ExoY (27). This is expected because the crystal structure was solved only after substantial proteolytic cleavage; as a result, roughly a third of the residues present in ExoY are absent in the structure. Consistent with the notion that the structure of proteolytically cleaved ExoY is smaller in size, the pair distribution ($P(r)$) calculated from the ExoY crystal structure is shifted left compared with that derived from the experimental SAXS profile of ExoY. Thus, our SEC-SAXS data support the previous assertion that ExoY exists as a monomer in solution.

Our data show that ExoY is a monomer in solution, and a previous mutational study suggested that ExoY possesses only one F-actin–binding region, the C-terminal end of ExoY (5). We therefore hypothesize that ExoY likely dimerizes to promote F-actin bundling, a mechanism commonly used by many cellular F-actin–bundling or cross-linking proteins, as discussed above. We propose that the monomeric ExoY binds F-actin, and this interaction stimulates a conformational change in ExoY that is a prerequisite for dimerization. This change would allow ExoY monomers bound to two separate actin filaments to come together and form a dimer. As one ExoY dimer is formed, it becomes easier for ExoY monomers bound to the same actin filaments nearby to dimerize. This process would cooperatively induce the formation of a stable two-filament bundle and could con-
tinue to promote the formation of larger, multifilament bundles.

To visualize how such a complex might form, we performed protein interface surface analysis (PISA) on the ExoY structure (PDB code 5XNW) (27). Like other bacterial toxins, anthrax EF and pertussis CyaA, ExoY consists of two domains, named catalytic domain A and B (CA and CB). Together, CA and CB form the catalytic center for nucleotidyl cyclase activity (Fig. 4B). Whereas this structure has several large loop regions missing, our PISA analysis revealed a potential dimerization interface between the CB domain of one ExoY subunit and the CA/CB interface of the other subunit (Fig. 4B), with a buried surface of 585 Å² and a ΔG of −3.7 kcal/mol.

Earlier mutational work revealed that a highly conserved Asp-25 residue in actin and the C-terminal region of ExoY play critical roles in mediating interaction between ExoY and F-actin (5, 6). We thus hypothesize that these regions are involved in the contact surface between F-actin and ExoY. Due to the missing C-terminal region of ExoY in the crystal structure, precise docking of ExoY and F-actin cannot be achieved. We therefore used actin Asp-25 as a landmark, to manually dock the C-terminal region within the CA domain of our synthetic ExoY dimer into an actin trimer based on charge complementarity (Fig. 4C). Putting together the ExoY dimer with the ExoY–F-actin trimer complex, we then generated a model for a 2:2 ExoY–F-actin complex, where both ExoY subunits use the same region of their respective CA domains to interact with the Asp-25 region of F-actin (Fig. 4D). This complex was then used to generate a molecular model for ExoY induced actin bundles (Fig. 4E). Our model can explain why ExoY is activated by binding F-actin, but not G-actin, because ExoY binds a cleft at the interface between actin subunits within the filament (6).

Support for the proposed model for ExoY bundling

Our model predicts that the putative ExoY-binding site on F-actin would overlap with the F-actin-binding sites of fimbrin and cofilin (Fig. 5A) (35, 36). As ExoY was demonstrated to bind and bundle F-actin with similar affinity to fimbrin, we then tested whether ExoY could compete with either fimbrin or cofilin for binding to preassembled actin filaments in high-speed sedimentation assays. As shown in Fig. 5 (B and C), ExoY was able to effectively compete with both fimbrin and cofilin for binding to F-actin. These results are consistent with our model that the F-actin-binding site for ExoY overlaps with that of fimbrin or cofilin.

Using our model, we then identified several residues that are predicted to mediate key interactions necessary for ExoY dimerization and, by extension, F-actin bundling. These residues were mutated, and the resulting constructs were purified and tested for their ability to bundle F-actin in low-speed sedimentation assays. Our model predicts that two residues, Glu-175 and Glu-177, may hamper dimerization due to potentially unfavorable interactions with residues Glu-76 and Asp-183 from the adjacent subunit. When these residues were mutated to serine, the resulting construct displayed a remarkable ~30-fold increase in its ability to bundle F-actin, despite having no change in its affinity for binding F-actin (Fig. 6A), although there was no detectable shift in the oligomeric state in the absence of F-actin, as the SEC elution profiles for WT ExoY and ExoY_E175S/E177S were indistinguishable (data not shown).

Our model also predicted that His-104 may play a role in mediating dimerization via charged interactions with Glu-45 from the adjacent subunit. His-104 is located near the missing N-terminal region at the dimer interface of the ExoY crystal structure, which contains a signal sequence for type III secretion (2). When this residue was mutated to serine, the resulting

Figure 5. ExoY competes with fimbrin and cofilin for binding to F-actin. A, comparison of cofilin (PDB code 5YU8) (56), fimbrin (PDB code 3BYH) (57), and ExoY-binding sites on F-actin. High-speed (100,000 × g) sedimentation demonstrates that increasing concentrations of HaloTagged ExoY displace 1 μM cofilin or 1 μM SNAP-tagged fimbrin from preassembled actin filaments (1.5 μM). B, representative gels showing that the amount of cofilin and SNAP-tagged fimbrin pelleted decreases as the amount of ExoY increases. C, relative pellet composition. Following SDS-PAGE and Coomassie Blue staining, cofilin or SNAP-tagged fimbrin pellet band densities were quantified and compared with the pellet band density for HaloTagged ExoY. All pellet band densities were normalized to the amount of actin in the pellet as a loading control. Error bars, S.E. of two independent replicates.
construct exhibited a substantially reduced ability to bundle F-actin, although it retained the ability to effectively bind F-actin (Fig. 6B). As the SEC elution profiles between WT and H104S mutant were indistinguishable, we concluded that there were no alterations in the solubility or oligomeric state of this construct compared with WT ExoY.

Asp-156 was also targeted for mutagenesis, as our model predicts the formation of a salt bridge between it and Arg-141 from the adjacent subunit. Mutation to serine produced a construct that was able to bind and bundle F-actin similar to WT ExoY (Fig. 6C). This mutant construct also produced an SEC elution profile consistent with WT ExoY and the other mutants described above. Together, our data support the notion that the putative ExoY dimerization interface is critical for F-actin bundling.

Discussion

Belyy et al. (6) showed that ExoY inhibits the rate of F-actin depolymerization using a bulk pyrene-based fluorescence assay in vitro. Additionally, ExoY intoxication increases the intracellular F-actin content in HeLa cells (6). However, the molecular basis underlying these phenomena remain unknown. Our TIRFm, sedimentation, and EM data demonstrate that ExoY can facilitate F-actin bundling. Our competition data, with fimbrin and coflin, also provide support for our model wherein ExoY binds at the interface of actin subunits within the filament. Furthermore, our mutational data strongly suggest that ExoY dimerization mediates F-actin bundling, as increasing the affinity for ExoY dimerization produced a stronger bundling protein, whereas weakening the putative ExoY dimerization interface produced a less efficient bundling protein. Taken together, our data support the notion that ExoY represents a novel class of F-actin–bundling proteins. ExoY-mediated F-actin bundling offers a molecular basis for how ExoY can inhibit F-actin depolymerization and increase cellular F-actin content (6).

ExoY is a well-characterized, promiscuous nucleotidyl cyclase whose enzymatic activity is stimulated by binding to F-actin, a critically important host cell protein (6–8). The dynamics of F-actin assembly have been well-established, and previous results have demonstrated that a catalytically inactive mutant of ExoY can stabilize actin filaments (6, 10). Our current studies provide the molecular basis for the interaction between ExoY and F-actin. Our results indicate that ExoY is
able to directly bundle F-actin, independent of its enzymatic activity. It is likely that the F-actin–bundling activity of ExoY is responsible for the previously observed stabilization of actin filaments, as similar observations have been made for other F-actin–bundling proteins (37, 38). P. aeruginosa has previously been demonstrated to stimulate the formation of membrane projections, called blebs, which form an intracellular niche supporting P. aeruginosa growth. The T3SS and ExoY were identified as key mediators of this lifestyle (24, 35, 39). As membrane blebs are known to be devoid of actin, the F-actin–bundling activity of ExoY could be responsible for this phenotype. CyaA, an adenyl cyclase toxin homologous to ExoY produced by B. pertussis, is known to stimulate formation of similar membrane projections in erythrocytes due to a localized increase in cAMP near the membrane (36). ExoY injection into the host cell likely establishes a concentration gradient, where the concentration of ExoY is higher local to the membrane and diminishes as the distance from the injection point increases. This suggests that ExoY may reach its critical bundling concentration first near the membrane, causing it to congregate at the cell cortex and exert a local effect similar to CyaA, which integrates into the cellular membrane.

Whereas enzymatic activity is not a prerequisite for F-actin bundling, we observed distinct differences in the physiology of F-actin bundles formed by WT versus catalytically inactive ExoY. We hypothesize that the conformational flexibility resulting from the various stages of cAMP generation may allow for a greater degree of freedom in terms of bundling geometry. This would impart to the WT ExoY-actin filament bundles a certain degree of flexibility and allow them to better handle stress forces applied to the bundle. In support of this theory, when we attempted to visualize the ExoY-actin filament bundles with higher resolution using cryo-EM, the ExoYK81M–actin filament bundles fell apart during vitrification, but the WT ExoY-actin filament bundles remained intact. It was shown that F-actin bundles exhibit distinct variations in their mechanical properties dependent upon the bundling protein (16). In particular, two types of F-actin bundle bending have been reported: decoupled bending, where each filament in the bundle bends independently, and fully coupled bending, where the filaments in the bundle behave in a similar fashion, as though they were glued together. It has been suggested that in decoupled bending bundles, the cross-linking protein does not resist shearing and is able to freely tilt, whereas in fully coupled bending bundles, the cross-linking protein strongly resists shear (16, 40). This would seem to suggest that the conformational heterogeneity associated with active catalysis in WT ExoY imparts a certain degree of structural elasticity to the bundle, which allows it to better accommodate the harsh forces imposed during vitrification, in essence allowing the WT ExoY bundles to bend but not break, whereas the structural rigidity imposed by the catalytically inactive ExoYK81M results in the bundles falling apart during vitrification. Thus, we suggest that, although ExoY enzymatic activity is not required for F-actin bundling to occur, the conformational heterogeneity associated with catalysis impacts the physical properties of the resulting actin bundle. We believe that an ExoY dimer best allows for this flexibility compared with a potential higher-order oligomer.

Thus, we further postulated that F-actin bundling is mediated by the cooperation of ExoY dimers, the formation of which is stimulated by the binding of ExoY to F-actin. It is possible that ExoY may utilize the proposed dimerization interface to assemble into higher-order oligomers. However, the modest cooperativity found in the F-actin high-speed sedimentation assay ($n = 1.37$) and the tight bundles observed by EM make this less likely. Future structural or biophysical analysis await to address this hypothesis.

The discovery that ExoY can bundle actin filaments raises interesting questions as to its mechanism of activation. Our laboratory has previously characterized the mechanisms by which two adenyl cyclases similar to ExoY, EF and CyaA, are activated by binding to the host calcium sensor, calmodulin (21, 41, 42). Using the insights from these structures, it would be tempting to infer that ExoY is activated via a similar mechanism. The regions of EF and CyaA that mediate interaction with calmodulin correspond with the region of ExoY predicted to mediate interaction with F-actin in our model. One of these regions, corresponding to residues 221–267 of ExoY, is absent in the crystal structure. In EF and CyaA, these residues, called switch A, undergo dramatic conformational changes upon binding calmodulin and include residues that stabilize the nucleotide binding pocket. It is known that these residues are naturally flexible in the absence of calmodulin and prone to proteolytic cleavage. Therefore, it is also logical to speculate that activation of ExoY follows a mechanism similar to that previously described for EF and CyaA: F-actin binding stabilizes several key loop regions, including switch A, which form the catalytic pocket and result in enhanced enzymatic activity. Different from the other cyclase toxins, ExoY may possess a two-step mechanism of activation. In the first step, F-actin binding stabilizes switch A, following the mechanism described for EF and CyaA. However, in contrast to EF and CyaA, actin-stimulated ExoY dimerization causes conformational changes that further stabilize the catalytic pocket and further activates the enzymatic activity of ExoY. Additional structural and mutational studies will explore this hypothesis.

ExoY-mediated F-actin bundling would allow activated ExoY to be sequestered from its host competitors. When ExoY is injected into a host cell, it faces stringent competition from hundreds of host ABPs. Our data indicate that ExoY possesses an average affinity for F-actin, meaning that it can be easily crowded out by excess host ABPs binding to actin filaments, preventing enzyme activation. However, our data also indicate that ExoY bundles F-actin with greater efficiency than it binds F-actin. We have also shown that ExoY forms extremely tight bundles, much tighter than many host-bundling proteins. As a result, ExoY within the bundle would remain associated with F-actin for a longer period of time compared with its unbundled counterparts, producing more cyclic nucleotide over the same time period. In this way, we believe that ExoY–F-actin–bundling activity functions to enhance ExoY catalysis in vivo, without activating the enzyme.

ExoY bears no structural or sequential homology to any previously characterized F-actin–bundling proteins. Therefore, we conclude that ExoY represents a novel family of F-actin–bundling proteins. To our knowledge, there are
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Currently three proteins of bacterial origin, aside from ExoY, which have been demonstrated to directly bundle actin filaments: Salmonella SipC, Burkholderia BimA, and Chlamydia Tarp (43–45). BimA possesses WH2 domains, which mediate F-actin bundling. WH2 domains are well-characterized actin-binding domains that are found in a number of eukaryotic ABPs (46). Like ExoY, the Chlamydia Tarp proteins and Salmonella SipC bear no homology to previously characterized F-actin–bundling proteins. However, both proteins have been demonstrated to dimerize, and this dimerization event has been suggested to mediate F-actin–bundling activity in both cases (44, 45, 47). Given our findings on ExoY, we suggest that ExoY, SipC, and Tarp represent a group of convergently evolved ABPs, unique in structure and function, which mediate actin bundling via dimerization.

P. aeruginosa is one of the most common pathogens causing respiratory infection in hospitalized patients, yet the bacterium is also capable of infecting other tissues, such as the eye and gut (1, 2). This pathogen uses a multitude of virulence factors to counteract host defenses and gain a fitness advantage. ExoY is one of four T3SS effectors that contribute significantly to the pathogenesis of this bacterium and thus represents a potential therapeutic target (3, 7, 8). Our work on the F-actin–bundling activity of ExoY offers new insights into how the action of ExoY may be inhibited. As ExoY represents a novel class of F-actin–bundling protein that disrupts cellular function via the production of cyclic nucleotides and bundling F-actin, the inhibition of both activities will be required. The ExoY dimerization interface and the ExoY–F-actin interaction interface both serve as potential targets to disrupt the toxic effects of ExoY.

P. aeruginosa has emerged as a model organism for the study of extracellular pathogens. However, P. aeruginosa has also been shown to invade host cells and replicate intracellularly (25, 35, 39). Although the mechanisms underlying P. aeruginosa invasion are unknown, manipulation of the host cell cytoskeleton is known to mediate invasion in well-characterized intracellular pathogens such as Burkholderia, Chlamydia, and Salmonella (43–45). P. aeruginosa remains relatively understudied as an intracellular pathogen, yet this bacterium has gained notoriety for its rapid development of antibiotic resistance and the difficulty in treating nosocomial P. aeruginosa infections (48). Thus, it is fair to wonder if a degree of this resistance stems from an intracellular subpopulation of P. aeruginosa that are not exposed to therapeutics, in addition to the conventional mechanisms of resistance (1, 49). It is known that P. aeruginosa infections resist antimicrobial therapy, in part, due to the rise of persister cells (50). Whereas most studies into P. aeruginosa persistence have focused on biofilms, we suggest that intracellular populations of P. aeruginosa may represent an underappreciated source of persister cells. Future studies await to explore the link between ExoY’s ability to bundle F-actin, the ability of P. aeruginosa to invade host cells, and the rate at which persister cells develop.

Experimental procedures

Protein expression and purification

An expression vector for P. aeruginosa ExoY (pET23ExoY) was graciously provided by Dara Frank (7). All point mutations were generated via site-directed mutagenesis using the QuikChange Lightning SDM kit (Agilent Technologies). HaloTag constructs were generated by cloning the exoY gene into the HindIII and EcoRI sites of the plasmid pKV1141. All constructs were verified via DNA sequencing. E. coli BL-21 (DE3) cells carrying the expression vector were grown in 2× YT medium with 100 μg/ml ampicillin at 37 °C with shaking at 225 rpm. Protein expression was induced at an A600 of ~0.7 via the addition of 100 μM isopropyl 1-thio-β-D-galactopyranoside at 30 °C with shaking at 175 rpm for 18 h. 1 mM benzamidine and 0.3 mM PMSF were then added to the culture, and the cells were harvested via centrifugation at 10,000 × g for 20 min. Resulting cell pellets were frozen and stored at −80 °C until needed.

Cell pellets were resuspended in 20 mM Tris, pH 7.5, 500 mM NaCl, 5 mM β-mercaptoethanol, 2 mM DTT, 5 mM imidazole, 1 mM benzamidine, 0.3 mM PMSF and lysed via sonication. Following lysis, additional benzamidine and PMSF were added, and the cell lysate was clarified via centrifugation at 140,000 × g for 30 min at 4 °C. The resulting supernatant was filtered and applied to a Source Q anion-exchange column pre-equilibrated with buffer containing 20 mM Tris-HCl, pH 7.7, 5 mM imidazole, 500 mM NaCl, 2 mM DTT. Bound protein was washed to baseline and eluted with buffer containing 20 mM Tris-HCl, pH 7.7, 250 mM imidazole, 500 mM NaCl, 2 mM DTT. The eluate was diluted to a NaCl concentration <50 mM and applied to a Source Q anion-exchange column pre-equilibrated with buffer containing 20 mM Tris-HCl, pH 8.0, 2 mM DTT. Bound protein was washed to baseline and eluted with a linear gradient of buffer containing 20 mM Tris-HCl, pH 8.0, 2 mM DTT, 500 mM NaCl. ExoY-containing fractions were pooled, concentrated, and run through a S200 size exclusion column pre-equilibrated with buffer containing 20 mM Tris-HCl, pH 8.0, 250 mM NaCl, 2 mM DTT, 2 mM CaCl2, 6 mM MgCl2. All proteins yielded a single elution peak, and protein purity was assessed via SDS-PAGE. Samples were aliquoted, flash-frozen in liquid nitrogen, and stored at −80 °C until needed. No discernible loss of function or stability was observed between frozen and unfrozen samples.

Actin was purified from rabbit acetone powder, as described previously (51). For TIRFm, actin was labeled with amine-reactive Alexa Fluor 488–succinimidyl ester (Life Technologies, Inc.) (28). For two-color experiments, ExoY was labeled with Cy3-reactive Alexa Fluor 555 maleimide (Life Technologies) per the manufacturer’s instructions. Cofilin and SNAP-tagged fimbrin were purified as described (52).

TIRFm

TIRF microscopy was performed following the detailed protocol outlined previously (28). Briefly, microscope slides (no. 1.5, Fisher) were coated with mPEG-silane and assembled into chambers. Mg-ATP–actin (1.5 μM, 15% Alexa Fluor 488–actin) was mixed with ExoY or the control buffer in a polymerization mixture containing 10 mM imidazole, pH 7.0, 50 mM KCl, 1 mM
MgCl₂, 1 mM EGTA, 50 mM DTT, 0.2 mM ATP, 50 μM CaCl₂, 15 mM glucose, 20 μg/ml catalase, 100 μg/ml glucose oxidase, and 0.5% (w/v) methylcellulose 400 centipoise and transferred to a chamber. TIRFM images were collected at 5-s intervals with an ixOn EMCCD camera (Andor Technology) using an Olympus IX-71 microscope equipped with through-the-objective TIRFm illumination or a cellTIRF 4Line system (Olympus). Frames were collected every 5 s, over a course of at least 20 min/experiment.

**EM**

20 μM G-actin was added to a polymerization mixture containing 10 mM imidazole, pH 7.0, 50 mM KCl, 1 mM MgCl₂, 1 mM EGTA, and incubated at 25 °C for 1 h. 5 μM F-actin was then mixed with an equimolar ratio of ExoY and incubated for 30 min at 25 °C. Following incubation, 3 μl of the ExoY–F-actin mixture was applied to an EM grid (400-mesh Cu, 01814-F, Ted Pella Inc.), stained with 0.75% uranyl formate and imaged using a 120-kV FEI Spirit electron microscope.

Samples for cryo-EM were prepared similarly as negatively stained samples. Preassembled F-actin was mixed with ExoY with final concentrations of 10 μM each and co-incubated for 30 min at 25 °C, after which 1.5 μl was applied to a grid (Quantifoil R 1.2/1.3 200-mesh Cu). The grid was blotted and plunged-frozen using an FEI Vitrobot (blot force 1, blot time 2.5 s, humidity 90%, wait time 15 s). Micrographs were collected on a 200-kV FEI Talos cryo-electron microscope.

**Sedimentation**

Sedimentation assays were performed as described (28). F-actin was polymerized as described above. HaloTagged ExoY and F-actin, at their indicated concentrations, were co-incubated for 30 min at 25 °C. Following co-incubation, samples were spun for 20 min at 10,000 × g (low speed) or 100,000 × g (high speed). For low-speed assays, 15 μl of the resulting supernatant was analyzed via SDS-PAGE. For high-speed assays, the supernatant was carefully removed, so as not to disturb the pellet, and 15 μl was analyzed via SDS-PAGE. The pellet was thoroughly resuspended in 150 μl of 1× hot protein sample buffer, and 15 μl was analyzed via SDS-PAGE. Competition assays contained 1 μM cofilin or SNAP-tagged fimbrin (from fission yeast) as indicated. Gels were stained with Coomassie and imaged on an Odyssey imaging system (LI-COR Biosciences). Quantification of band intensity was performed in ImageJ.

**SEC-SAXS**

SEC-SAXS data were collected at the BioCAT/18ID beamline at the Advanced Photon Source, Argonne National Laboratory (Chicago, IL), using the photon-counting PILATUS 3 M at room temperature (23 °C) and an incident X-ray wavelength of 1.03 Å. The 3.5-m sample-to-detector distance yielded a range of 0.005–0.33 Å⁻¹ for the momentum transfer (q = 4π sinθ/λ, where 2θ is the scattered angle between the incident and scattered beam and λ is the X-ray wavelength). 2.5 mg of ExoY was loaded onto a GE Superdex 200 10/300G in the buffer containing 20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 2 mM CaCl₂, 6 mM MgCl₂, 2 mM DTT, 0.1 mM ATP, and the eluates were immediately passed through the X-ray beam. The SAXS data reduction and analyses were performed using BioXTAS RAW (53). Comparison of experimental scattering profiles with calculated profiles from high-resolution atomic models was done using CRYSO (54).

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