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Capping of actin filaments by vinculin activated by the *Shigella* IpaA carboxyl-terminal domain☆

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Abstract ***Shigella*, the causative agent of bacillary dysentery, invades epithelial cells. Upon bacterial–cell contact, the type III bacterial effector IpaA binds to the cytoskeletal protein vinculin to promote actin reorganization required for efficient bacterial uptake. We show that the last 74 C-terminal residues of IpaA (A559) bind to human vinculin (HV) and promotes its association with actin filaments. Polymerisation experiments demonstrated that A559 was sufficient to induce HV-dependent partial capping of the barbed ends of actin filaments. These results suggest that IpaA regulates actin polymerisation/depolymerisation at sites of *Shigella* invasion by modulating the barbed end capping activity of vinculin.**

Keywords: IpaA; Vinculin; Actin; Capping; *Shigella*

1. Introduction

Shigella, a gram-negative bacillus known as the major etiological agent of dysentery in developing countries, mediates its entry into epithelial cells by a trigger-like process involving a type III secretion apparatus and injection of several bacterial type III effectors [1,2]. Bacterial entry into host cells requires the dynamic polymerisation and depolymerisation of actin, which serves to mould the contact site to allow efficient entry of the pathogen [2]. The type III effector IpaA binds to vinculin, and the IpaA–vinculin complex induces actin depolymerisation, which is required for effective bacterial entry [3,4].

Vinculin, a highly conserved cytoskeletal component of focal adhesions, plays critical roles in various processes such as cellular adhesion and motility, as well as in embryonic development and tumorigenesis [5–7]. Vinculin is comprised of five α-helical bundle domains that form a large globular head, and a five-helical bundle tail (Vt) domain, which is connected to the head via a proline-rich hinge region [8]. The isolated head domain of vinculin binds to talin and α-actinin, while the isolated tail domain binds F-actin [9,10]. However, in its native state an intramolecular interaction between the N-terminal seven-helical bundle domain (Vh1) of vinculin with its Vt tail domain clamps vinculin in a closed conformation, and masks its binding sites to some of its ligands such as F-actin and paxillin [11]. The binding of the vinculin-binding sites (VBSs) present in the central rod domains of talin or α-actinin with the Vh1 domain induces conformational changes that displace the head–tail interaction and are alone sufficient to trigger vinculin binding to F-actin, a hallmark of vinculin activation [11–13]. Here we report that the 74-residues C-terminal domain of IpaA activates vinculin binding to actin and mediates vinculin-dependent capping of the barbed end of actin filaments.

2. Materials and methods

2.1. Antibodies

The rabbit anti-IpaA antiserum was previously described [3]. The mouse anti-vinculin monoclonal antibody Vin11.5, and the rabbit anti-actin were from Sigma Corp (St. Louis, MO). The anti-rabbit IgG HRP antibody was from Amersham Corp.

2.2. Bacterial strains and plasmid constructs

S. flexneri strains are derivatives of the wild-type strain M90T [14]. Strain SF635, in which the *ipaA*, *B*, *C* and *D* genes have been deleted (Δ*ipa*) was used for purification of IpaA [15]. Bacteria were grown in tryptic casein soy broth (TCBS) at 37 °C. To obtain the IpaA derivatives AΔ1, AΔ2 and AΔ3, internal deletions of the *ipaA* gene in the plasmid pBad18::ipaA [16] were performed between residues 69 (*Bsu36I*) to 139 (*PshA1*) (AΔ1), 139 (*PshA1*) to 420 (*StuI*) (AΔ2) and 419 (*StuI*), as well as a C-terminal deletion from residues 552–633 (*SnaBI*) (AΔ3). The Δ*ipa* strain was transformed with plasmid pBad18::AΔ1 (Δ*ipa*-AΔ1), pBad18::AΔ2 (Δ*ipa*-AΔ2) or pBad18::AΔ3 (Δ*ipa*-AΔ3) or with pBad18::ipaA (Δ*ipa*-IpaA). A559 is a fusion protein containing GST fused to the C-terminal residues 513–633, or 560–633 of IpaA, respectively, expressed from a derivative of pGEX-4T2 in *E. coli*.

2.3. Purification of proteins and overlay assays

IpaA was purified from cultures of a Δ*ipa*/pBad: IpaA strain as previously described [4]. GST-N and A559 fusions were purified using glutathione beads, according to the manufacturer's instructions.

☆ While this paper was submitted, a work by Demali et al. reported that the first 500 amino-terminal residues of IpaA could induce vinculin-independent actin depolymerisation following cell transfection [24].

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Human recombinant vinculin was purified from *E. coli* as described [8]. Vinculin overlay assays were performed as previously described [3,17].

2.4. Actin sedimentation assays

Recombinant vinculin (1.8 μM) and IpaA or the A559 derivative (200, 400 or 800 nM), were added to 3 μM G-actin (cytoskeleton) during the polymerisation reaction in F-buffer (2 mM Tris pH 7.5, 0.2 mM $CaCl_2$, 0.2 mM ATP, 100 mM KCl and 2 mM $MgCl_2$) for 1 h at room temperature (RT). Proteins were centrifuged at 70000 × *g* for 30 min in a TL-100 ultracentrifuge (Beckman). Pellet and supernatant fractions were analysed by SDS–PAGE and Coomassie staining.

2.5. Polymerisation assays using pyrenyl-actin fluorescence

Actin was purified from rabbit muscle and labelled on cysteine 374 by pyrenyl iodoacetamide [18,19]. For measurements of the initial rate of barbed end growth, samples of 2 μM actin (10% pyrenyl-labelled) containing 0.2 nM spectrin-actin seeds were supplemented with vinculin (0–20 μM) and/or A559 (0–15 μM). Polymerisation was started by addition of 100 mM KCl, 0.2 mM EGTA and 1 mM $MgCl_2$ to the solution in G-buffer. For measurements of dilution-induced depolymerisation at barbed ends, 25 μM actin (75% pyrenyl labelled) was polymerised by addition of 1 mM $MgCl_2$, 0.2 mM EGTA and 100 mM KCl for 20 min at RT. F-actin was then diluted to 67 nM in F buffer and supplemented with vinculin (0–2 μM) and/or A559 (0–2 μM). The initial rate of decrease in fluorescence of pyrenyl-F-actin was measured. Changes in fluorescence were recorded using a Safas flx spectrofluorometer. The excitation and emission monochromators wavelengths were set at 366 nm and 407 nm, respectively.

3. Results

3.1. The C-terminus of IpaA is sufficient to reveal the latent F-actin binding potential of vinculin

To identify the domain of IpaA that mediates its interactions with vinculin overlay assays were performed. We generated IpaA derivatives containing internal deletions between residues 69–139 (AΔ1) and 139–420 (AΔ2), carboxyl-terminal deletions of residues 419–633 (AΔ3) and 559–633 (AΔCT), as well as a GST-fusion protein containing IpaA C-terminal residues 560–633 (A559) (Fig. 1A). These IpaA derivatives were expressed and migrated at their expected sizes in SDS–PAGE (Fig. 1B). Full length IpaA, the internal IpaA deletions AΔ1 and AΔ2, as well as the C-terminal fusion A559, all bound to full-length human vinculin (HV, Fig. 1C). However, the AΔ3 and AΔCT mutant IpaA proteins failed to bind to HV (Fig. 1C).

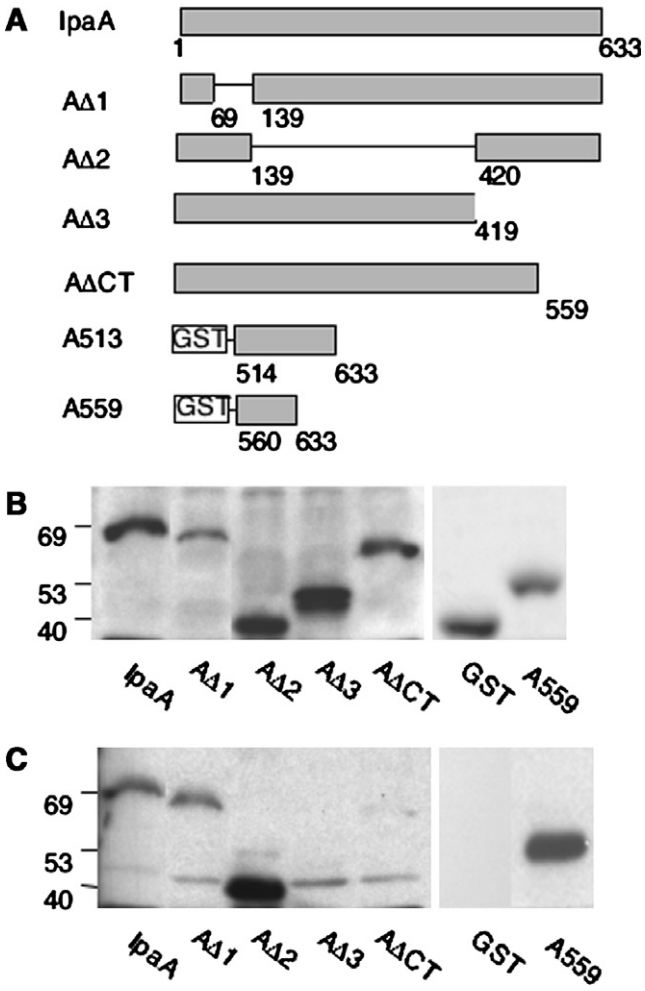

Fig. 1. The 74 C-terminal residues of IpaA mediate binding to vinculin. (A) The structure of IpaA derivatives containing either internal deletions between residues 69–139 (AΔ1), 139–420 (AΔ2), 419–633 (AΔ3), or 559–633 (AΔCT), or GST fused to the C-terminal residues 514–633 (A513) or 560–633 (A559) of IpaA is shown. (B) Supernatants from Δ*ipa*-IpaA, AΔ1, AΔ2, AΔ3, AΔCT *Shigella* strains, and purified GST and A559, were analysed by SDS–PAGE and anti IpaA-immunoblotting. A559 was detected by staining with Coomassie blue. (C) Binding of the IpaA derivatives to vinculin was tested in overlay assays.

To address the role of the IpaA vinculin-binding domain in regulating vinculin–F-actin interactions, actin sedimentation assays were performed with the A559 and HV proteins. As expected, when HV (1.8 μM) and F-actin (3 μM) were incubated together the majority of the actin pool was recovered in its polymerised form in the pellet fraction whereas HV was mostly found in the soluble fraction (Fig. 2A). Addition of A559, however, led to a significant shift of HV into the pellet fraction with polymerised actin (Fig. 2A). Quantification by scanning densitometry indicated that the percentage of the total pool of HV associated with F-actin shifted from 13% in the absence of A559 to 86% in the presence of 2 μM A559 (Fig. 2B).

Therefore, the last 74 C-terminal residues of IpaA are sufficient to induce the association of vinculin with actin filaments.

3.2. A559–HV induces vinculin-dependent partial barbed-end capping of actin filaments

To analyse the effects of the A559–vinculin interaction on actin dynamics, the rate of barbed end growth of actin filaments was measured by monitoring changes in pyrenyl-labeled actin fluorescence. Effects of barbed end elongation were studied using spectrin-actin seeds, which are short actin filaments capped at their pointed ends. When A559 was added at concentrations ranging from 0 to 875 nm to HV at a concentration of 2 μM, partial inhibition of actin polymerisation was observed, plateauing at 40% inhibition at saturating amounts of A559 (>800 nM, Fig. 3A). A similar limited 40% inhibition was observed at higher concentrations of HV (8 μM) in the presence of 10 μM A559, demonstrating that the partial inhibition of barbed ends did not result from partial saturation of barbed ends (not shown). When the reverse experiments were performed in which increasing concentrations of HV were added to 0.9 μM of A559, a partial inhibition of actin polymerisation was again observed, with 40% inhibition occurring at concentrations of HV greater than 1.8 μM (Fig. 3B, inset). In contrast, neither HV alone nor A559 alone affected the ini-