Biochemical and Biophysical Research Communications 420 (2012) 816–821

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Chlamydia trachomatis Tarp cooperates with the Arp2/3 complex to increase the rate of actin polymerization

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ARTICLE INFO

Article history:
Received 22 February 2012
Available online 23 March 2012

Keywords:
Chlamydia trachomatis
Tarp
Arp2/3 complex
Actin

ABSTRACT

Actin polymerization is required for *Chlamydia trachomatis* entry into nonphagocytic host cells. Host and chlamydial actin nucleators are essential for internalization of chlamydiae by eukaryotic cells. The host cell Arp2/3 complex and the chlamydial translocated actin recruiting phosphoprotein (Tarp) are both required for entry. Tarp and the Arp2/3 complex exhibit unique actin polymerization kinetics individually, but the molecular details of how these two actin nucleators cooperate to promote bacterial entry is not understood. In this study we provide biochemical evidence that the two actin nucleators act synergistically by co-opting the unique attributes of each to enhance the dynamics of actin filament formation. This process is independent of Tarp phosphorylation. We further demonstrate that Tarp colocalization with actin filaments is independent of the Tarp phosphorylation domain. The results are consistent with a model in which chlamydial and host cell actin nucleators cooperate to increase the rate of actin filament formation.

1. Introduction

Chlamydia trachomatis is the most prevalent sexually transmitted bacterium in the United States [1]. Chlamydia can infect the genital tract of both men and women. A majority of infections in women are asymptomatic, which if not detected, can lead to pelvic inflammatory disease, ectopic pregnancy and infertility [2]. Worldwide, *C. trachomatis* infection of the eye (trachoma) is the leading cause of preventable blindness with an estimated 40 million active trachoma infections in 2009 [3].

Chlamydiae display a unique developmental cycle in which the extracellular infectious elementary bodies (EBs) invade human epithelial cells. Once internalized the EB differentiates into a reticulate body (RB) within a membrane bound vacuole called an inclusion and undergoes several rounds of replication before differentiating back to EBs, which are released from the infected cell and can initiate a new infection [4,5].

C. trachomatis entry into a human cell is dependent on host cytoskeletal rearrangements triggered by bacterial attachment to the host cell surface [6]. Host cells treated with drugs such as cytochalasin D that disrupt actin polymerization inhibit chlamydial entry [6]. In addition, chlamydial entry is inhibited by disruption of the host actin related proteins 2 and 3 nucleating (Arp2/3) complex [7,8]. A chlamydial type III secreted effector protein termed Tarp, for translocated actin recruiting phosphoprotein, has also been implicated in the bacterial induced cytoskeletal changes that permit EB entry [9]. Tarp harbors distinct actin binding and oligomerization domains which allow for the clustering of multiple actin monomers to nucleate a new actin filament [10]. Antibodies with specificity to the Tarp actin binding domain, when microinjected into host cells prior to *C. trachomatis* infection, inhibited bacterial entry [11]. Taken together, these findings implicate both host and bacterial actin nucleation complexes in driving cytoskeletal changes required for *C. trachomatis* entry. The molecular details of Tarp and Arp2/3 complex interactions are unknown.

Each of these two independent actin nucleators employs unique strategies for the generation of new actin filaments. The host cell Arp2/3 complex is comprised of seven proteins including the actin related proteins 2 and 3 polypeptides which share homology with actin [12–14]. The Arp2/3 complex is regulated by host cell machinery such as those members of the Wiskott–Aldrich syndrome family proteins (WASP) whose members are themselves regulated by signal transduction cascades [15,16]. The Arp2/3 complex associates with existing actin filaments to nucleate a new actin filament forming a branch at an angle of approx. 70°

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http://dx.doi.org/10.1016/j.bbrc.2012.03.080

[12]. Conversely, Tarp is a single chlamydial polypeptide of 1005 amino acids, which harbors distinct phosphorylation, oligomerization and actin binding domains [9,10,17]. Tarp is able to associate with globular actin to nucleate the formation of linear actin filaments without activation from a nucleation promoting factor [10].

C. trachomatis Tarp is rapidly phosphorylated by host tyrosine kinases such as Abl, Syk and Src family members following translocation into the host cell [18,19]. Phosphorylated Tarp has been shown to associate with phosphoinositide 3-kinase (PI3K) and Src homology 2 (SH2) domain containing transforming protein 1 (SCH-1) via their respective SH2 domains [20]. Additionally, phosphorylated Tarp had been suggested to be implicated in the GTPase mediated activation of the host cell Arp2/3 complex [20]. Consequently, a role for Tarp phosphorylation in bacterial entry is controversial as chemical inhibitors which prevent Tarp phosphorylation do not inhibit chlamydial entry [18]. Furthermore, Tarp orthologs from other chlamydial species are not phosphorylated suggesting they have evolved mechanisms to circumvent any requirement for Tarp phosphorylation [11,17,21].

We demonstrate here that Tarp and the Arp2/3 complex cooperate to increase the rate of actin polymerization and were accompanied by a concomitant increase in branched actin filaments observed by scanning electron microscopy. We show that Tarp phosphorylation does not alter the cooperation between Tarp and the Arp2/3 complex. Furthermore, Tarp colocalization with actin filaments is independent of the Tarp phosphorylation domain. Taken together our results indicate that bacterial and host cell derived actin nucleators work cooperatively to increase the rate of actin filament formation and promote chlamydial entry.

2. Materials and methods

2.1. SDS–PAGE and immunoblotting

Proteins were separated on SDS-10% polyacrylamide gels and stained with coomassie R-250 (Pierce) or transferred to 0.45 μm pure nitrocellulose transfer and immobilization membrane (Schleicher & Schuell, Keene, NH). Immunoblotting employed peroxidase conjugated secondary antibodies (Chemicon International, Temecula, CA) and Supersignal West Pico chemiluminescent substrate (Pierce, Rockford, IL). The anti-phosphotyrosine 4G10 monoclonal antibody was purchased from Upstate (Millipore). Polyclonal rabbit antibodies directed towards *C. trachomatis* L2 LGV 434 Tarp was developed at Rocky Mountain Laboratories as previously described [9].

2.2. Cloning, protein expression and purification

An in frame glutathione-S-transferase (GST) and polyhistidine *C. trachomatis* L2 LGV 434 Tarp fusion protein was generated by PCR amplifying the corresponding coding regions from *C. trachomatis* genomic DNA (QIAGEN genomic purification kit, Valencia CA) using custom synthesized oligonucleotide primers (Integrated DNA Technologies, Coralville, IA) engineered with SalI, SacI or NotI linkers. PCR products were purified (QIAGEN), digested with restriction enzymes (New England Biolabs, Beverly, MA) and subcloned into linearized pGEX-6P-1 to generate translational fusions with GST at the N-terminus and polyhistidine at the C-terminus. The PCR fragments described above harboring *tarP* were also cloned into pEGFP-C3 (BD Biosciences Clontech) to allow for the ectopic expression of eGFP-Tarp in HeLa cells.

pGEX-6P-1 plasmids were transformed into BL21 strain of *Escherichia coli* (Novagen, Madison WI). Protein expression and purification were performed according to the procedures outlined for Ni Sepharose 6 Fast Flow and Glutathione Sepharose 4B in the Bulk GST Purification Module (GE health sciences, Piscataway, NY).

2.3. Pyrene assay

The rate of actin polymerization in the presence of Tarp and the Arp2/3 complex was monitored according to the methods outlined in the Actin Polymerization Biochem Kit BK003 (Cytoskeleton, Denver CO). Briefly, monomeric pyrene labeled actin was prepared by diluting 500 μg of lyophilized pyrene actin into 5 mls of 5 mM Tris (pH 8.0), 0.2 mM $CaCl_2$, and 0.2 mM ATP (G-buffer) and incubating for 1 h at room temperature followed by an additional hour of incubation at 4 °C. Monomeric pyrene actin was obtained by collecting the supernatant following a 2 h, 100,000 rcf, 4 °C spin in a Beckman Optima TLX Ultracentrifuge using a TLA 100.3 rotor (Beckman Colter Inc., Fullerton, CA). Approximately 40 μg of pyrene labeled actin was gently mixed with 2–5 μg of test proteins in a volume of 500 μl for 5 min prior to the addition of 1/20th volume of polymerization buffer (500 mM KCl, 20 mM $MgCl_2$, and 10 mM ATP). The reaction was monitored over one hour with an LS 55 Luminescence spectrophotometer equipped with a biokinetics accessory and directed by FL WinLab software version 4.0 (Perkin Elmer, Beaconsfield, BUCKS, UK) with 2.5 nm bandwidth at 365 nm excitation wavelength and 2.5 nm bandwidth at 407 nm emission wavelength.

2.4. Scanning electron microscopy

Actin filaments were added to an SEM type 3 mount and sputter coated with 8–10 nm chromium. Coated filaments were examined in a Hitachi S5200 scanning electron microscope at 30 kV accelerating voltage.

2.5. Transfection of HeLa cells and indirect immunofluorescence microscopy

Hela cells (2×10^5) were seeded in 6 well plates with coverslips and grown for 24 h in DMEM containing 10% FBS. Cells were then transfected with transfection mixture containing 8 μl of Fugene HD (promega) and 2.5 mg of respective plasmid. Following 24 h, cells were fixed by adding 4% paraformaldehyde and incubating at 4 °C for 15 min. Cells were then treated with ice cold 0.4% Triton-X for 10 min, followed by blocking with 5% BSA for 45 min. To visualize tyrosine phosphorylated protein, cells were first incubated with anti-phosphotyrosine primary antibody (upstate) at 1:1000 dilutions in 0.5% BSA at RT for 45 min followed by incubation with anti-mouse secondary antibody conjugated to Alexa 350 (invitrogen). To simultaneously visualize actin, phalloidin conjugated to Alexa 568 (invitrogen) was added to the above mixture containing secondary antibodies. To stain for Arp2/3 complex, cells were first incubated with anti-Arp3 primary antibody (upstate) at 1:100 dilutions in 0.5% BSA at RT for 45 min followed by incubation with anti-rabbit secondary antibody conjugated to Alexa 594 (invitrogen). Coverslips were rinsed and mounted in Prolong Gold antifade reagent (invitrogen). Cells were examined with a Zeiss Axio Observer A1 microscope equipped with phase contrast and epifluorescence optics. Images were obtained using an AxioCam MRm camera controlled by AxioVision 4.8.2 and further processed using Adobe Photoshop CS2.

3. Results

3.1. Tarp and activated Arp2/3 cooperate to polymerize actin

We have previously shown that the actin filaments produced by chlamydial Tarp are linear [10], whereas actin filaments produced via activated Arp2/3 complex from existing filaments result in branched actin filaments [13]. To test whether the chlamydial