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Heparin-binding hemagglutinin HBHA from *Mycobacterium tuberculosis* affects actin polymerisation

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ABSTRACT

HBHA is a mycobacterial cell surface protein that mediates adhesion to epithelial cells and that has been implicated in the dissemination of *Mycobacterium tuberculosis* (*Mtb*) from the site of primary infection. In this work, we demonstrate that HBHA is able to bind G-actin whereas its shorter form, deprived of the lysine-rich C-terminal region (HBHAΔC), does not bind. Consistently, interaction of actin with HBHA is competitive with heparin binding. Notably, we also observe that HBHA, but not HBHAΔC, clearly hampers G-actin polymerisation into F-actin filaments. Since *Mtb* escapes from the phagosome into the cytosol of host cells, where it can persist and replicate, HBHA is properly localised on the bacterial surface to regulate the dynamic process of cytoskeleton formation driven by actin polymerisation and depolymerisation.

1. Introduction

Mycobacterium tuberculosis (*Mtb*) is one of the deadliest human pathogens. Key to tuberculosis (TB) pathogenesis is the ability of the bacilli to survive in harsh conditions [1], replicate in host cells and disseminate from the site of primary infection to potentially any organ, where it can persist for decades and eventually reactivate to cause active TB [2–4]. Dissemination from the site of primary infection involves interactions of *Mtb* with epithelial cells through a virulence factor called heparin-binding haemagglutinin (HBHA) [5–8]. HBHA is a surface exposed protein, that mediates binding of mycobacteria to epithelial cells through its C-terminal lysine rich domain, which interacts with heparan sulphate proteoglycans at the cell surface [7,9,10]. In a previous work, we have determined the Small Angle X-ray Scattering (SAXS) solution structure of this molecule and showed that it exhibits a dimeric and elongated shape, with C-terminal lysine-rich domains protruding towards the same side of the protein [11,12]. This structure is consistent with the role attributed to the HBHA C-terminus in driving the binding of bacteria by establishing electrostatic interactions with epithelial heparan sulphate proteoglycans [7,10]. Besides being responsible for epithelial cell adhesion [7], HBHA can cross epithelia cell layers and enter the cytoplasm [5]. Once inside the cytosol, HBHA, but not its truncated form (HBHAΔC) induces a reorganisation of actin cytoskeleton [5]. This finding is very important in light of the newly recognised ability of *Mtb* to escape from the phagosome into the cytosol, where it replicates and cause cell damage that is instrumental for bacterial spread and infection of other cells [13]. Indeed, HBHA-deficient strains are hampered in their ability to disseminate from the lungs to other tissues [14].

Actin, one of the most abundant proteins in eukaryotic cells, readily assembles into long helical filaments to form the cytoskeleton. Cells continuously control the growth and shrinkage of actin filament networks in order to perform tasks crucial for their survival such as cell motion, cell division, and phagocytosis [15,16]. Moreover, various microbes harness the actin polymerisation machinery through their surface proteins to allow intracellular motility within host cells and subsequent dissemination [17]. *Mycobacterium marinum* can escape from phagosomes and recruit host cell cytoskeletal factors in the cytoplasm to induce actin polymerisation resulting in intracellular motility and direct cell to cell spread [18,19]. Similarly, *Mtb* is ejected from the infected cells through an actin-based structure, denoted as ejectosome [20], although no evidence of *Mtb* actin tail formation in the cytosol of infected cells has hitherto been found [13].

Using Single Molecule Force Spectroscopy (SMSF), it was shown that both HBHA and HBHAΔC are able to bind actin [21], a finding that does not explain why only full-length HBHA is able to induce cytoskeleton reorganisations [5]. We here analysed the actin binding capabilities of both HBHA and HBHAΔC and the possible causes which allow HBHA (i) to efficiently bind actin and (ii) to alter the actin filament network. To this aim, we studied actin filament nucleation and polymerisation, two crucial steps in intracellular

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organelle movements [16], in response to HBHA and HBHAΔC. Results provide an explanation for the ability of HBHA to affect cytoskeleton morphology and strengthen the concept that HBHA is involved in *Mtb* pathogenesis at different dissemination levels.

2. Materials and methods

2.1. Sample preparation

HBHA and HBHAΔC were cloned, expressed and purified as previously reported [11]. Skeletal muscle actin, purchased from Sigma, was dissolved in water and dialysed against G buffer (5 mM Tris–HCl pH 8.0, 0.2 mM $CaCl_2$, 0.2 mM ATP, 1 mM DTT) overnight at 4 °C. The G buffer ensures a monomeric state of G-actin. The dialysed sample was concentrated to prepare a stock solution of 16.2 μM, which was properly diluted either for surface plasmon resonance or fluorescence studies. For fluorescence studies, pyrene actin (purchased from Cytoskeletron) was dissolved in the G buffer at a final concentration of 25 μM.

2.2. Surface plasmon resonance studies

Actin was immobilised in 10 mM acetate buffer pH 4.5 (flow rate 5 μL/min, time injection 7 min) on a CM5 Biacore sensor chip, using EDC/NHS chemistry, following the manufacturer's instructions [22]. Residual reactive groups were deactivated with 1 M ethanolamine hydrochloride, pH 8.5; the reference channel was prepared by activating with EDC/NHS and deactivating with ethanolamine. Experiments were carried out at a 25 °C, at constant 20 μL/min flow rate using as running buffer, a solution of Hepes 10 mM, pH 7.4, NaCl 150 mM, $CaCl_2$ 2 mM, surfactant P200.05% v/v (90 μL injected for each experiment). Binding experiments were carried out using HBHA at various concentrations in the range of 0.1–70.0 μM and HBHAΔC concentrations 1, 10 and 100 μM. All experiments performed in triplicate.

The BIA evaluation analysis package (version 4.1, GE Healthcare, Milano, Italy) was used to subtract the signal of the reference channel and to estimate K_D values. RUmax data *versus* proteins concentrations were fitted by non-linear regression analysis with GraphPad Prism, vers. 4.00, GraphPad Software (San Diego, California). K_D values were also estimated using the BIA evaluation v.4.1 software. Competitive SPR experiments with heparin were carried out pre-incubating HBHA at a fixed concentration of 3.0 μM with increasing concentrations of heparin ranging from 0 to 5.4 μM using an average molecular weight, 5000 Da, for heparin polymer.

2.3. G-actin polymerisation studies

16.2 μM skeletal muscle actin was dialysed against G buffer overnight at 4 °C, while pyrene actin (Cytoskeletron) was freshly prepared 25 μM in the same buffer. Both solutions were treated with EGTA and $MgCl_2$ at final concentration 1 and 0.2 mM, respectively, and left on ice for 1 h. G-actin and pyrene actin were mixed in molar ratio 1:12, incubated 2 h at 4 °C and centrifuged at 100,000 rpm in TLA120 rotor for 30 min. The supernatant was collected, aliquoted and stored at −80 °C. Actin polymerisation (6 μM, 7.7% pyrene labelling) was induced by addition of 10× polymerisation buffer (0.5 M KCl, 20 mM $MgCl_2$, 20 mM ATP, 0.5 M KHepes pH7.0, 0.75 M Imidazole) and pyrene fluorescence was monitored by exciting at 365 nm and measuring emission at 420 nm. Five minutes after polymerisation start, parallel experiments were carried out by adding HBHA 10, 30 and 60 μM and HBHAΔC 60 μM. The effect of the inhibitor latrunculin B on actin polymerisation was measured by adding 2 μM latrunculin B 8 min after polymerisation start. As control experiments, fluorescence was measured after the sole addition of the polymerisation buffer. In addition, to check that polymerisation was induced solely by the polymerisation buffer, fluorescence was also measured on actin samples with no addition of the polymerisation buffer.

2.4. G-actin nucleation studies

For nucleation experiments of G-actin polymerisation, 4% pyrene G-actin sample was incubated in the 10× buffer (0.4 M KCl and 20 mM $MgCl_2$) for 5 min. Following incubation, pyrene fluorescence was monitored by exciting at 365 nm and measuring emission at 420 nm. Three minutes after measurement start, two parallel experiments were carried out by addition of either HBHA 20 or 50 μM. As a positive control of G-actin nucleation, further experiments were performed after addition of 1 and 2 μM of the known G-actin nucleator polylysine (average length of 24 residues, Sigma). A further control was made by measuring pyrene fluorescence of G-actin samples without addition (of either HBHA or polylysine).

2.5. Electrostatic potential calculations

The crystal structure of the uncomplexed G-actin in the ADP state (PDB code 1j6z) was used for the computation of G-actin electrostatic potential surface [23]. Electrostatic surface computation was performed using the software GRASP [24].

3. Results

3.1. HBHA interacts with actin solely through its C-terminus

Binding of both HBHA and HBHAΔC (lacking residues 161–199, Fig. 1A), prepared as reported [11], to skeletal muscle G-Actin was checked by Surface Plasmon Resonance (SPR). Actin was immobilised on the chip surface to an overall immobilisation level of 350 RU. For the estimation of the dissociation constants, analytes concentrations were employed in the range between 0 and 70 μM. As a result, SPR experiments revealed a clear association of actin with HBHA (Fig. 1B). These experiments were carried out by injecting HBHA solutions at increasing concentrations using a 20 μL/min flow rate. Divalent cations (2 mM $CaCl_2$) were added to the running buffer to ensure a monomeric organisation of actin [25].

Kinetic experiments along with RUmax value of each experiment *versus* HBHA concentration, both employing a 1:1 interaction model, provided a low micromolar dissociation value for G-actin–HBHA complex. In detail, best fitting of experimental data, by a non-linear regression analysis, resulted a K_D of 9.0 ± 0.9 μM (Fig. 1B). Similar experiments, carried out on HBHAΔC, unambiguously showed that this truncated protein is unable to bind actin, even when high HBHAΔC concentrations are used. Indeed, no variation of RUmax values is registered up to 100 μM HBHAΔC concentration (Fig. 1C).

3.2. HBHA interacts with actin using the same region involved in heparin binding

SPR experiments suggested that HBHA interacts with actin through its C-terminal arm (Fig. 1). In order to corroborate this finding, we set up competition experiments using heparin as competitor [26]. Indeed, it was previously shown that the C-terminus of HBHA is able to bind heparin similar to heparan sulphates of epithelial cells [9]. Therefore, we checked whether heparin binding interferes with HBHA–actin interactions. SPR competition experiments