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Cyclophilin A is the potential receptor of the *Mycoplasma genitalium* adhesion protein

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ABSTRACT

The *Mycoplasma genitalium* adhesion protein (MgPa), the most important outer membrane protein of *M. genitalium*, plays a vital role in the adhesion to and invasion of host cells by *M. genitalium*. Identification of MgPa receptors will help elucidate the pathogenic mechanism of *M. genitalium*. However, the receptor protein of MgPa has not been reported to date. In this study, an MgPa-binding protein with a molecular weight of approximately 17 kDa was screened from SV-HUC-1 cell membrane proteins by a modified virus overlay protein binding assay (VOPBA). Liquid chromatography-mass spectrometry (LC–MS) was used to analyze the protein components of the 17-kDa protein. The results demonstrated that the MgPa-binding protein was most likely Cyclophilin A (CyPA). The binding activity and distribution of CyPA in SV-HUC-1 cells were detected using indirect ELISA, western blotting, far-western blotting and indirect immunofluorescence. We found that recombinant MgPa (rMgPa) could bind with CyPA from SV-HUC-1 cell membrane proteins and to recombinant CyPA, which indicated that CyPA was predominant component of the 17-kDa protein band and can interact with rMgPa. In addition, an indirect immunofluorescence assay showed that CyPA was partially distributed on the membrane surfaces of SV-HUC-1 cells and could partially inhibit the adhesion of rMgPa and *M. genitalium* to SV-HUC-1 cells. Co-localization assays further indicated that rMgPa and *M. genitalium* can interact with CyPA. These results suggested that the CyPA located on SV-HUC-1 cell membranes may be the potential receptor of MgPa, which could provide an experimental basis for elucidating the function of MgPa and the possible pathogenic mechanism of *M. genitalium*.

1. Introduction

Mycoplasma genitalium, with a genome size of only 580 kbp, was first isolated in 1981 and is associated with sexually transmitted diseases (STDs) (Fraser et al., 1995; Taylor-Robinson, 2002; Tully et al., 1981). *M. genitalium* infection is usually chronic, and while the symptoms are inconspicuous or absent at the early stage (Wang et al., 1997), this infection can cause acute and chronic non-gonococcal urethritis (NGU) in men (Kikuchi et al., 2014) and genital tract diseases in women, such as bacterial vaginitis, pelvic inflammatory disease (PID) (Haggerty and Taylor, 2011; Moller et al., 1984), endometritis and infertility (Cohen et al., 2002; Rajkumari et al., 2015). *M. genitalium* is also one of the pathogens associated with opportunistic infection in individuals infected with human immunodeficiency virus (HIV) and is one of the synergistic pathogens of HIV (Dehon et al., 2016). MgPa is the main membrane protein of *M. genitalium* and is crucial for the formation of the tip structure and for the adhesion properties of *M. genitalium*. This protein plays a pivotal role in the diagnosis and prevention of *M. genitalium*. The interaction between MgPa and the receptor of MgPa, located on the cell membranes of host cells, is critical to the pathogenicity of *M. genitalium*. *M. genitalium* must adhere to the surfaces of epithelial cells and colonize the site of infection or invade host cells before having pathogenic effects on the host. *M. genitalium* can adhere to the cells in an MgPa-mediated manner; however, the MgPa mutant that lacks adhesion activity also lacks infectivity toward host cells (Burgos et al., 2006).

M. genitalium receptors are one or more cell membrane proteins that bind specifically with *M. genitalium* and mediate the adhesion of host cell invasion of *M. genitalium* and are the determinants of host range and tissue tropism of *M. genitalium* infection. *M. genitalium* adheres to or even invades host cells via the binding of MgPa with the corresponding receptors located on the membrane surface of the host cell, resulting in

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pathogenic effects on the host. Because the adhesion of *M. genitalium* to host cells is performed mainly by MgPa, the MgPa receptor is the primary receptor involved in adhesion of and invasion by *M. genitalium*.

The virus overlay protein binding assay (VOPBA), based on specific binding between viruses and their corresponding receptor proteins, is a classical method for the identification of virus receptors. Li et al. confirmed that sialic acid is the receptor of the bovine adenovirus serotype 3 by using the VOPBA technique (Li et al., 2009). HSP70, the cell receptor of Japanese encephalitis virus, was also identified by a VOPBA (Das et al., 2009). In this study, the modified VOPBA was performed to screen for MgPa-binding proteins from the membrane proteins of SV-HUC-1 cells. The cell membrane proteins extracted by ultrasonic fragmentation were transferred to a PVDF membrane, which was then incubated with purified rMgPa, and the target band was identified using liquid chromatography-mass spectrometry (LC–MS). The potential receptor was verified by indirect ELISA, western blotting, far-western blotting and an indirect immunofluorescence assay. In addition, we also performed immunofluorescence co-localization assays of CyPA and rMgPa and of CyPA and *M. genitalium* in SV-HUC-1 cells, which further indicated CyPA can interact with MgPa. The results of this study are helpful for the identification of the MgPa receptor and further understand the pathogenic mechanism of *M. genitalium* infection, laying a theoretical foundation for using receptor analog molecules or antagonists as new target drugs and vaccines to prevent and treat *M. genitalium* infection.

2. Materials and methods

2.1. Antibodies and reagents

The recombinant *M. genitalium* adhesion protein (rMgPa; amino acids 1075–1444, containing the main immunodominant epitope region) was purified using Ni-nitrilotriacetic acid (NTA) beads, and the corresponding rabbit anti-rMgPa polyclonal antibody was purified by affinity chromatography using CNBr-activated Sepharose 4B as described in our previous study (Zeng et al., 2012). Cy3-conjugated AffiniPure goat anti-rabbit IgG (H + G) (Cat. SA00009-2, USA) and fluorescein (FITC)-conjugated AffiniPure goat anti-mouse IgG (H + L) (Cat. SA00003-1, USA) were purchased from Protein-tech. 4, 6-Diamidine-2-phenylindole dihydrochloride (DAPI) (Cat. C0065, China) and 3, 3′, 5, 5′-tetramethylbenzidine (Cat. PR1200, China) were purchased from Solarbio Life Sciences. Recombinant human CyPA was purchased from ProSpec (enz-359, Israel). Rabbit anti-CyPA antibody (ab41684, UK) and mouse anti-CyPA antibody (ab58144, UK) were purchased from Abcam Corporation.

2.2. Cell culture and propagation of Mycoplasma genitalium

The SV40 immortalized human urothelium cell (SV-HUC-1) (ATCC, CRL-9520) containing the SV40 genome were purchased from Chinese Academy of Sciences Cell Bank (shanghai, China) and were cultured in 1 × F-12 K nutrient mixture (Kaighn's modification) (Gibco, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, Australia) and penicillin-streptomycin solution (100U/mL penicillin, 100 μg/mL streptomycin) (Beyotime, CAS C0222, China) at 37 °C in a humidified CO_2 (5%) incubator. After the SV-HUC-1 cells covered 80% of the bottom of the cell culture flask (Corning, USA), the adherent cells were dissociated with 0.25% trypsin-EDTA (Gibco, CAS 25200-056, USA) and transferred to 24-well plates (Corning, USA) with sterile glass coverslips. The cell density was adjusted to 2×10^4 cells/mL of medium, and 500 μL of the cell suspension was added to each well. The plates were incubated for 24 h at 37 °C in a humidified CO_2 (5%) incubator.

M. genitalium G37, which was purchased from ATCC and stored in our laboratory, was cultured in SP-4 medium at 37 °C in tissue culture flasks (Corning, USA) (Tully et al., 1979). After growth for 5 days, the color of the medium changed from red to orange, which indicated exponential growth, and the *M. genitalium* was collected at this stage. The *M. genitalium* cells attached to the bottom of the culture flask was scraped off in phosphate-buffered saline (PBS, pH 7.4) and pelleted by centrifugation at 10,000 × *g* for 30 min. The precipitate was resuspended in 4 mL of PBS and centrifuged in Eppendorf tubes at 10,000 × *g* for 15 min. The *M. genitalium* cells were then resuspended in 4 mL of F-12 K nutrient mixture supplemented with penicillin-streptomycin solution (100U/mL penicillin, 100 μg/mL streptomycin).

2.3. Extraction of cell membrane proteins by ultrasonic fragmentation

Cell membrane proteins were prepared as described by Grogan DW (Grogan, 1996). Briefly, 10^7 SV-HUC-1 cells were pelleted by centrifugation at 1000 × *g* for 5 min, and the cell pellet was collected. The cell pellet was gently washed three times using PBS, which was followed by centrifugation at 1000 × *g* for 5 min. The cell lysate was prepared by homogenization for 20 min in ice-cold lysis solution with a protease inhibitor cocktail containing PMSF. Ultrasonic fragmentation (10 W; ultrasonic pulse, 5 s; interval, 15 s; a total of 30 cycles) was performed, and the supernatant was collected after centrifugation at 2500 rpm for 10 min. Then, the supernatant was centrifuged again at 16,000 × *g* for 30 min, and the pellet was collected and resuspended with 300 μL of PBS. Membrane protein concentration was measured by the Bicinchoninic Acid (BCA) Protein Assay Kit.

2.4. Screening of cell membrane proteins that specifically bind with rMgPa using a modified VOPBA

The extracted SV-HUC-1 cell membrane proteins were separated by SDS-PAGE and transferred to a PVDF membrane (Corning, USA) using a semi-dry transfer unit. The PVDF membrane was blocked overnight with 5% skim milk powder at 4 °C, which was followed by three washes with TBS containing 0.5% Tween-20(TBST) and incubation with rMgPa (2 μg/mL) for 6 h at 4 °C. Then, the PVDF membrane was first incubated with anti-rMgPa antibody (1:1000) for 2 h at 37 °C after three washes with TBST and then incubated with HRP-conjugated goat anti-rabbit IgG antibody (1:5000) for 1 h at 37 °C after three washes with TBST. The PVDF membrane was washed five times with TBST and then visualized using a chemiluminescent imaging system (Syngene, USA).

2.5. Target band with a molecular weight of 17 kDa was identified using LC–MS

The target bands were identified using a liquid chromatography-mass spectrometry (LC–MS) instrument by Huijun Biotechnology Co. Ltd. (Guangzhou, China).

2.6. Binding assay between rMgPa and recombinant CyPA using indirect ELISA

The wells of polystyrene microtiter plates (Corning, USA) were coated with 150 μL/well of rMgPa (100 μg/mL), and the plates was incubated overnight at 4 °C. The plates were then washed three times with TBST and then blocked with 200 μL/well of BSA (5 mg/mL) in 0.1 mol/L $NaHCO_3$ (pH 8.6) for 2 h at 4 °C. The wells were washed three times followed by a 2-h incubation at 37 °C with 100 μL/well of recombinant CyPA protein (1:1000). Then, the wells were washed three times before addition of 100 μL/well of rabbit anti-CyPA antibody (1:1000), and the plates were then incubated for 2 h at 37 °C. Simultaneously, as a positive control, 100 μL of rabbit anti-rMgPa antibody (1:1000) was transferred to the corresponding wells, and the plates were then incubated for 2 h at 37 °C. The plates were washed five times with TBST, which was followed by addition of HRP-conjugated goat anti-rabbit IgG antibody (1:5000) and incubation for 1 h at 37 °C. After the plates were washed six times with TBST, color development