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Mycoplasma fermentans deacetylase promotes mammalian cell stress tolerance

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A B S T R A C T

Mycoplasma fermentans is a pathogenic bacterium that infects humans and has potential pathogenic roles in respiratory, genital and rheumatoid diseases. NAD^+-dependent deacetylase is involved in a wide range of pathophysiological processes and our studies have demonstrated that expression of mycoplasmal deacetylase in mammalian cells inhibits proliferation but promotes anti-starvation stress tolerance. Furthermore, mycoplasmal deacetylase is involved in cellular anti-oxidation, which correlates with changes in the proapoptotic proteins BIK, p21 and BIM. Mycoplasmal deacetylase binds to and deacetylates the FOXO3 protein, similar with mammalian SIRT2, and affects expression of the FOXO3 target gene BIM, resulting in inhibition of cell proliferation. Mycoplasmal deacetylase also alters the performance of cells under drug stress.

This study expands our understanding of the potential molecular and cellular mechanisms of interaction between mycoplasmas and mammalian cells.

1. Introduction

Mycoplasmas, bacteria that do not produce a cell wall, cause diseases in plants, humans and other animals (Baranowski et al., 2014; Biondi et al., 2014; Kristensen et al., 2014; Taylor-Robinson, 2014; Whitson et al., 2014) and are frequent contaminants of laboratory cell cultures (Hasebe et al., 2013). *Mycoplasma fermentans* is a cell-invasive mycoplasma that can attach and enter human cells, and has potential pathogenic roles in respiratory and genital infections (Liu et al., 2012; Rawadi, 2000; Razin et al., 1998; Rottem, 2003) as well as rheumatoid arthritis (Kawahito et al., 2008; Wright, 2011). *M. fermentans* even can fuse with a variety of cells, after which the proteins are released and promote phosphorylation of cellular constituents via interaction with serine/threonine and tyrosine protein kinases cascades. The high fusogenicity of *M. fermentans* with Molt-3 cells is apparently stimulated by Ca^{2+} and depends on the proton gradient across the mycoplasma cell membrane (Dimitrov et al., 1993; Rottem, 2003). However, mycoplasma-related cellular and molecular mechanisms have not been comprehensively investigated to date (Díaz-García et al., 2006; Yavlovich et al., 2004).

Certain mycoplasmal proteins play important roles in interactions between mycoplasmas and host cells, such as attachment, entry and intracellular localization (Nakane et al., 2011; Pich et al., 2009; Thomas et al., 2013), or between mycoplasmas and the host immune system (Grover et al., 2014). Protein acetylation and deacetylation are common post-translational modifications that play critical roles in many important biological processes (Eskandarian et al., 2013). Some deacetylases in bacteria such as CobB, LpxC and NaxD, may be involved in metabolism and biosynthesis (Liu et al., 2014a) or catalyze the committed step of lipopolysaccharide (LPS) biosynthesis (Clayton et al., 2013; Zeng et al., 2013). Other deacetylases are required for important modifications of the outer membrane component lipid A in *Francisella* (Llewellyn et al., 2012) or are involved in deacetylating acetylated sugars on the bacterial cell membrane to allow surface adhesion and biofilm formation or to prevent recognition by the host immune system (Strunk et al., 2014; Wan et al., 2013). In addition, certain deacetylases, such as *Streptococcus pneumoniae* N-acetylglucosamine-6-phosphate deacetylase, can be secreted into the environment or the host milieu (Choi et al., 2013a).

Thus far, no research has been performed on the functions of

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mycoplasmal NAD$^+$-dependent deacetylase, a SIR2 family-like protein. NAD$^+$-dependent protein deacetylases are involved in multiple cell processes including growth, differentiation and energy metabolism (Ji et al., 2016) as well as a wide range of pathophysiological processes including myocardial injury, Parkinson's disease, and Huntington's disease (Choi et al., 2014; Xie et al., 2016). Mycoplasmal NAD$^+$-dependent deacetylase (DeA) is produced by the human pathogen *M. fermentans* (Liu et al., 2012). In this study, we explored the effects of *M. fermentans* deacetylase on mammalian cells.

2. Materials and methods

2.1. Cells, antibodies and other reagents

Murine melanoma cell line B16F10 and human embryonic kidney cell line HEK293T were cultured in Dulbecco's Modified Eagle's Medium (DMEM). Human colon cancer cell line HCT116 was cultured in McCoy's 5A medium with 10% fetal bovine serum (FBS). Cell Counting Kit 8 (CCK-8) was purchased from Dojindo Laboratories (Kumamoto Technology Research Park, Japan). DMEM was purchased from Cellgro (Herndon, VA, USA). FBS, penicillin and streptomycin were purchased from HyClone (Logan, UT, USA). Puromycin was purchased from Gene Operation (Ann Arbor, MI, USA). The following antibodies were used: anti-FLAG (Sigma, Saint Louis, MO, USA), anti-GAPDH (ABclonal Biotech Co, College Park, MD, USA), anti-Erk1/2, -p53, -BIK, -BIM and -p21 (Cell Signaling Technology, Beverly, MA, USA), anti-HA and anti-myc (MBL International, Woburn, MA, USA), anti-GST (Santa Cruz, Dallas, TX, USA), anti-FOXO3 and anti-acetyl-lysine (Millipore, Billerica, MA, USA). The antibodies against *Mycoplasma fermentans* deacetylase (DeA) and ribosome-binding factor A (RbfA) were prepared by our laboratory.

2.2. M. fermentans culture

M. fermentans (JER strain) was cultured in modified G-PPLO medium (67.2% PPLO (w/v), 20% rabbit serum (v/v), 10% yeast extract (w/v), 1% glucose (w/v), 0.2% phenol red (w/v), 1.6% NaOH (w/v), and 1000 U of penicillin/ml) for one week, and the culture was then centrifuged for 30 min at 12,000 rpm to harvest the bacteria. The mycoplasmas and culture supernatant were analyzed by western blotting using anti-DeA and anti-RbfA antibodies.

2.3. Preparation of antibodies against mycoplasmal proteins

The deacetylase gene of *M. fermentans* was cloned into the pGEX-4T-1 plasmid for expression as a fusion protein, GST-DeA, and 50 μg of this protein was used for mouse immunization. The immunization was repeated three times continuously for three weeks, and the mice were sacrificed to obtain the polyclonal antibody anti-DeA. Western blots were used to screen the DeA-specific antibody. Briefly, the RbfA gene was cloned into the pGEX-4T-1 plasmid for expression as a fusion protein of GST-RbfA. The immunization and polyclonal antibody screening procedures were the same as that of producing anti-DeA. The animal experiments were approved by the Medical Ethics Committee of Peking University.

2.4. Development of cell lines

The nucleotide sequence of the *M. fermentans* JER strain deacetylase gene (DeA, GeneID: 9793339) was redesigned, synthesized and cloned into the plasmid pcDNA3.1 by Songon Biotech Co., Ltd, Shanghai, China. The recombinant plasmid was digested with *Bam*H I and *Eco*R I, and the resulting fragment was ligated into pMSCV-puro (digested with *Bgl* II and *Eco*R I) to generate pMSCV-Flag-DeA. pMSCV-Flag-DeA, pVSV-G and the helper plasmid were co-transfected into HEK293T cells, and packaged viruses were harvested and used to infect B16F10 and HCT116 cells. Deacetylase-expressing cell lines were obtained by puromycin screening. Briefly, the nucleic acid sequence of HA-SIRT2 was inserted into pMSCV-puro plasmid and the packaged viruses were used to infect HCT116 cells to obtain SIRT2-stably-expressed cell line by puromycin screening.

2.5. Cell proliferation assay

After counting, 1×10^3 pMSCV-Control or pMSCV-Flag-DeA (deacetylase) retrovirus-infected cells in DMEM with 10% FBS were seeded into 96-well microplates and incubated at 37 °C for four days. Cell viability was assayed using 90 μL of DMEM with 10% FBS and 10 μL of CCK-8 at 37 °C for 1 h. The absorbance at 450 nm was measured using a microplate reader.

2.6. Cell adhesion assay

After counting, 1×10^5 pMSCV-Control or pMSCV-Flag-DeA retrovirus-infected cells in DMEM with 10% FBS were seeded into 12-well microplates and incubated at 37 °C (1 h for B16F10 cells, 2 h for HCT116 cells). The cells were then washed with phosphate-buffered saline (PBS) (twice for B16F10 cells, once for HCT116 cells), and 180 μL of DMEM with 10% FBS and 20 μL of CCK-8 were added. After incubation at 37 °C for 1 h, cell viability was determined by measuring the absorbance at 450 nm using a microplate reader.

2.7. Oxidative stress test

After counting, 5×10^3 pMSCV-Control or pMSCV-Flag-DeA retrovirus-infected cells in DMEM with 10% FBS were seeded into 96-well microplates and incubated at 37 °C for 2 h. The medium was then replaced with DMEM containing 200 μM H_2O_2, and the cells were incubated at 37 °C for four days. Cell viability was analyzed using CCK-8 (Choi et al., 2010).

2.8. Starvation test

After counting, 5×10^3 pMSCV-Control or pMSCV-Flag-DeA retrovirus-infected cells in DMEM with 10% FBS were seeded into 96-well microplates and incubated at 37 °C for 2 h. The medium was then replaced with DMEM without FBS, and the cells were incubated at 37 °C for four days. Cell viability was analyzed using CCK-8.

2.9. Drug tolerance assay

After counting, 5×10^3 pcDNA3.1-Control or pcDNA3.1-Flag-DeA plasmid-transfected HCT116 cells in DMEM with 10% FBS were seeded into 96-well microplates and incubated at 37 °C for 5 h. The culture medium was then replaced with fresh medium containing 40 ng/mL paclitaxel (PTX) or 25 μg/mL 5-fluorouracil (FU), and the cells were maintained for four days. Cell viability was assayed with 90 μL of DMEM with 10% FBS and 10 μL of CCK-8 at 37 °C for 1 h. The absorbance at 450 nm was measured using a microplate reader.

2.10. Western blot analysis

After culturing, B16F10 or HCT116 cells were washed twice with PBS, lysed with lysis buffer (50 mM Tris-Cl, 1% sodium dodecyl sulfate (SDS), 10% glycerol, pH 6.8) on ice for 5 min, and heated at 95 °C for 20 min. SDS-PAGE (SDS-polyacrylamide electrophoresis) was performed using 50 μg of total proteins, which were then transferred to a polyvinylidene difluoride (PVDF) membrane. The PVDF membrane was blocked with 5% milk in Tris-buffered saline and Tween-20 (TBST) overnight at 4 °C or for 1 h at room temperature. The membrane was washed three times with TBST and then incubated with an anti-Flag antibody (M2, in TBST with 5% non-fat milk) at 4 °C overnight or at

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