Lactobacillus slpA promotes ESC growth through the ERK1/2 pathway

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Abstract Bacterial surface layers (S-layers) are cell envelope structures ubiquitously found in gram-negative and gram-positive bacteria, including *Lactobacillus*. S-layers play a role in the determination and maintenance of cell shape as virulence factors, mediate cell adhesion, and regulate immature dendritic and T cells. In this study, we sought to understand the involvement of MAPK serine/threonine kinases in alterations in Endometrial epithelial cells (ESC) growth induced by *Lactobacillus crispatus* (*L. crispatus*) slpA, an S-layer protein. We applied various concentrations of *L. crispatus* to cultured ESCs and observed growth and changes in the phosphorylation status of ERK1/2, JNK, and p38. Similar experiments were conducted using *L. crispatus* lacking and over-expressing slpA. We found that ESC growth was altered by slpA primarily via ERK1/2. Our findings suggest that *L. crispatus* slpA promotes ESC growth mainly through an ERK1/2-dependent pathway.

Keywords slpA · MAPK · ESC · Lactobacillus

Introduction

The endometrium is composed of luminal and glandular epithelial cells, stromal components, and a closely associated extracellular matrix (Kitaya and Yasuo 2013). Endometrial cells, especially luminal and epithelial cells, modulate the physiology of the uterus. Although endometrial proliferation is estrogen-driven, it is also mediated by a number of growth factors through autocrine and/or paracrine signaling (Kitaya and Yasuo 2013; Lei et al. 2006). Endometrial modulation is essential for the preservation of normal uterine physiology, and this modulation is driven by many factors (Lei et al. 2006).

Bacterial surface layers (S-layers) are cell envelope structures ubiquitously found in Gram-negative and Gram-positive bacteria, including *Lactobacillus* (Yang et al. 2016; Perras et al. 2015). S-layers are composed of identical glycoprotein subunits with a molecular mass in the range of 40 to 200 kDa. S-layer proteins self-assemble into two-dimensional crystalline structures with oblique, square, or hexagonal symmetry, covering the entire cell surface. The subunits are held together and attached to the underlying cell wall by noncovalent interactions and have an intrinsic ability to spontaneously form regular layers in solution and on solid supports (Farci et al. 2015; Sleytr et al. 2014). S-layers have been shown to determine and maintain cell shape

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as virulence factors, mediate cell adhesion, and regulate immature dendritic and T cells. Moreover, S-layers function as a protective coat, molecular sieve, murein hydrolase, and ion trap (Fagan and Fairweather 2014; Currie et al. 2014; Baneyx and Matthaei 2014).

Bacterial adhesion to epithelial and subepithelial tissue is an important initial event in successful colonization of the mammalian intestine and other tissue sites. Several adhesion molecules have been characterized for bacterial species that cause infectious diseases in humans or animals (Xiao et al. 2013; Quistgaard et al. 2012). However, knowledge is very limited regarding adhesion molecules present on the mammalian commensal genus *Lactobacillus*. S-layers have been found in several *Lactobacillus* species. S-layer protein genes from *L. brevis*, *L. helveticus*, and *L. acidophilus* have been sequenced. Sequence similarity between *Lactobacillus* S-layer protein genes is found only between closely related *Lactobacillus* species; the primary sequences of *Lactobacillus* S-layer proteins show extensive variability, with the number of identical amino acids varying from 7 to 100% (Bobeth et al. 2011; Vilen et al. 2009). As a group, *Lactobacillus* S-layer proteins differ from those of most other bacteria in their small size (25–71 kDa) and high calculated isoelectric point (pI) values (9.4–10.4). Further investigation of S-layer protein slpA at the molecular level is essential for the development of applications that take advantage of these characteristics (Vilen et al. 2009; Narita et al. 2006).

Most S-layer proteins can reassemble in physiological buffers to form a regular, insoluble array. The adhesive properties of such protein arrays have been analyzed by solid-phase assays using a soluble ligand. Here, we report the effects of *Lactobacillus crispatus* (*L. crispatus*) slpA on endometrial epithelial cells, as well as the molecular mechanisms mediating these effects.

Materials and methods

Unless otherwise specified, all chemicals and reagents in the study were purchased from the Sigma Chemical Company (St. Louis, MO, USA). Antibodies to IgG, GAPDH, ERK1/2, phospho-ERK1/2, p38, phospho-p38, RSK90, phospho-RSK90 JNK, and phospho-JNK were purchased from the Millipore Corporation (Billerica, MA, USA). U1026 was purchased from the Sigma Chemical Company.

Cell culture

Endometrial epithelial cells (ESCs, purchased from ScienCell, Carlsbad, CA, USA) were cultured at 38.5 °C with 5% CO₂ under humidified air.

Bacterial strains and cultivation conditions

L. crispatus strains (derived from a 30 year old female volunteer in our hospital) were grown in MRS broth (Sigma) at 37 °C.

Cell proliferation assay with the Cell Counting Kit-8

A Cell Counting Kit-8 (CCK-8) kit (Dojindo Laboratories, Kumamoto, Japan) was used to determine cell viability and proliferation. Briefly, the cells were seeded in a 96-well plate (3000 cells/well, four replicates) and incubated overnight. The cells were incubated with different concentrations of *L. crispatus* (0, 10/ml, 10²/ml, 10³/ml, 10⁴/ml). The cells were replenished with a medium containing CCK-8 solution (10 µL CCK-8 in 100 µL medium) and incubated for another 2 h, after which the absorbance at 450 nm was measured using a microplate reader (Bio-Tek Instruments, Winooski, VT, USA). The growth percentage = (12 h, 24 h, 46 h, and 48 h OD – 0 h OD)/0 h OD.

Western blot

For western blotting experiments, 30 µl of total protein was electrophoresed on a denaturing 12% SDS-PAGE gel and transferred overnight to a 0.45 µm nitrocellulose membrane at 70 mA using neutral Tris-glycine (TG) buffer. Membranes were blocked for 1 h at room temperature using 5% skim milk. Primary antibodies were incubated for 60 min in 0.5% skim milk in Tris-buffered saline (TBS) at room temperature with shaking. Membranes were washed three times with TBS-Tween (TBST; 50 mM Tris-Cl, 150 mM NaCl, and 0.1% Tween 20; pH 7.5) for 10 min. Membranes were incubated with secondary antibodies (goat anti-rabbit IgG conjugate; Roche, Indianapolis, IN, USA) for 30 min and subjected to four 15-min washes with TBST. Proteins were visualized using an ECL detection method.

Plasmid construction for slpA and ΔslpA

The methods of Narita were used to delete the slpA gene (Narita et al. 2006). All PCRs were carried out

using the KOD-Plus-polymerase (Toyobo, Osaka, Japan). The *slpA* gene from *Lactobacillus acidophilus* JCM 1132 was amplified by PCR. The native *slpA* and Δ *slpA* expression system (lacking *slpA*) were generated by PCR amplification. These amplified fragments were digested with BglII and EcoRV prior to introduction into the BglII and PvuII sites of pI α A22. The resulting plasmids were labeled pI α A22-*slpA* and pI α A22- Δ *slpA*. The nucleotide sequence analyses of the fragments inserted into all plasmids were performed using an Applied Biosystems, Inc. (ABI) Prism 310 genetic analyzer with a Big Dye terminator version 3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA). The plasmids were introduced into *L. crispatus* (Narita et al. 2006) by electroporation.

Statistical analyses

The SPSS (IBM Inc., Armonk, NY, USA) and StatView (SAS Inc., San Francisco, CA, USA) software packages were used for statistical analyses. Significance was determined using analysis of variance (ANOVA) to enable comparison between the continuous numerical data of multiple groups. The Protected Least Significant Difference test was used for posthoc analyses.

Results

Effect of *L. crispatus* on ESC growth and optimal *L. crispatus* concentration

The CCK-8 was used to assess the effects of 0, 10/ml, 10^2 /ml, 10^3 /ml, and 10^4 /ml *L. crispatus* on ESC growth after 12, 24, 36, and 48 h. ESC growth was directly related to time and the concentration of *L. crispatus*; maximum ESC growth was observed after 36 h of exposure to 10^3 /ml *L. crispatus* (Fig. 1).

Effect of *L. crispatus* on MAPK signaling in ESCs

The effect of 10^3 /ml *L. crispatus* on MAPK signaling in ESCs was assessed after 0, 20, 40, and 60 min. ERK1/2 phosphorylation increased over time and peaked at the 40 min time-point. However, JNK and P38 showed no changes in phosphorylation status (Fig. 2).

To confirm the role of ERK1/2 in the effects of *L. crispatus* on ESC growth, ERK1/2 phosphorylation

Fig. 1 ESC growth following exposure to different concentrations of *L. crispatus* (0, 10/ml, 10^2 /ml, 10^3 /ml, 10^4 /ml) for 12, 24, 36, and 48 h

was inhibited by U0126. JNK and P38 phosphorylation was also unchanged by inhibition of ERK1/2 phosphorylation (Fig. 3a, b). RSK90, a protein

Fig. 2 Influence of 10^3 /ml *L. crispatus* on MAPK signaling. **a** Expression levels of total and phosphorylated ERK1/2, JNKs, and p38. **b** Relative protein expression (phosphorylation/total) of ERK1/2, JNKs, and p38 based on western blotting. Results are presented as mean $\pm$ SD ($n = 5$). *Significantly different from the 0 min group ($P < 0.05$). #Significantly different from the 20 min group ($P < 0.05$)

downstream of ERK1/2 signaling, phosphorylation was unchanged by inhibition of ERK1/2 phosphorylation (Fig. 4a, b). Inhibition of ERK1/2 phosphorylation inhibited ESC growth in comparison with that of the control group (Fig. 4c).

Influence of slpA on ESC growth

The phosphorylation status of ERK1/2, JNK, and P38 was unchanged in ESCs exposed to *L. crispatus* lacking slpA (Fig. 5a, b); however, growth was inhibited in ESCs exposed to *L. crispatus* lacking slpA in comparison with that of the control group (Fig. 5c). In contrast, ERK1/2 phosphorylation was increased in ESCs exposed to *L. crispatus*

Fig. 3 Phosphorylation status of ERK1/2, JNK and p38 after ERK1/2 inhibition by U1026. **a** Expression of ERK1/2, JNK, p38, phosphorylated ERK1/2, phosphorylated JNK, and phosphorylated p38. **b** Relative protein expression (phosphorylation/total) of ERK1/2, JNK and p38 based on western blotting. Results are presented as mean $\pm$ SD ($n = 5$). *Significantly different from the 0 min group ($P < 0.05$). #Significantly different from the 20 min group ($P < 0.05$). con control group without any *L. crispatus* effect on the ESC cells

Fig. 4 Phosphorylation status of RSK90 after ERK1/2 inhibition by U1026. **a** Expression of RSK90 and phosphorylated RSK90. **b** Relative protein expression (phosphorylation/total) of RSK90 based on western blotting. Results are presented as mean $\pm$ SD ($n = 5$). *Significantly different from the 0 min group ($P < 0.05$). #Significantly different from the 20 min group ($P < 0.05$). **c** Growth of ESCs exposed to *L. crispatus* after 12, 24, 36, and 48 h

overexpressing slpA, with maximum phosphorylation after 20 min of exposure (Fig. 6).

Discussion

Lactic acid bacteria (LAB) are important industrial micro-organisms that are used to ferment dairy products and other foods to preserve them and enhance their texture. Furthermore it was the main probiotics in the female genital tract. Many studies have demonstrated the potential of LAB in protein production, but

Fig. 5 Influence of *L. crispatus* lacking slpA on MAPK signaling. **a** Expression of ERK1/2, JNK, p38, phosphorylated ERK1/2, phosphorylated JNK, and phosphorylated p38. **b** Relative protein expression (phosphorylation/total) of ERK1/2, JNKs, and p38 based on western blotting. Results are presented as mean $\pm$ SD ($n = 5$). *Significantly different from the 0 min group ($P < 0.05$). #Significantly different from the 20 min group ($P < 0.05$). **c** Growth of ESCs exposed to *L. crispatus* after 12, 24, 36 and 48 h. Control: *L. crispatus* contains the slpA gene, Knockout: *L. crispatus* without slpA gene

further studies are required to characterize the properties of LAB as hosts for the expression of heterologous and homologous gene products (Vilen et al. 2009; Narita et al. 2006). In this study, we found that *L. crispatus* promoted ESC growth through activation of the ERK1/2 branch of the MAPK pathway. The optimal *L. crispatus* concentration to promote growth

Fig. 6 Influence of *L. crispatus* overexpressing slpA on MAPK signaling. **a** Expression of total and phosphorylated ERK1/2, JNKs, and p38. **b** Relative protein expression (phosphorylation/total) of ERK1/2, JNKs, and p38 based on western blotting. Results are presented as mean $\pm$ SD ($n = 5$). *Significantly different from the 0 min group ($P < 0.05$). #Significantly different from the 20 min group ($P < 0.05$). **c** Growth of ESCs exposed to *L. crispatus* after 12, 24, 36, and 48 h. Control: *L. crispatus* contains the slpA gene, Over expression: *L. crispatus* with the slpA expression vector

was 10^3 /ml. Inhibition of ERK1/2 inhibited ESC growth under *L. crispatus* exposure.

Most bacterial S-layers are composed of a single protein species, the S-layer protein, which greatly varies in size among different bacterial genera (Perras et al. 2015; Fagan and Fairweather 2014; Babolmorad et al. 2014). S-layers are cell envelope structures ubiquitously found in gram-positive and gram-negative bacteria as well as in Archaea. S-layers self-assemble into two-dimensional crystalline structures with oblique, square, or hexagonal symmetry,

covering the entire cell surface. S-layers are hydrophobic and crystallize to form a two-dimensional layer on the bacterial surface (Vilen et al. 2009). The role of slpA in bacterial adhesion to chicken epithelium has been suggested previously. Here, we showed that slpA promoted ESC growth (Fagan and Fairweather 2014; Babolmorad et al. 2014; Sleytr et al. 2007). When the slpA gene was deleted, ERK1/2 phosphorylation in exposed ESCs was inhibited, whereas slpA overexpression induced ERK1/2 phosphorylation. The MAPKs are a super-family of serine/threonine kinases that include ERK, JNK, and p38 (Reus et al. 2014). MAPKs are involved primarily in the activation of nuclear transcription factors that control cell proliferation, differentiation, and apoptosis (Delhanty et al. 2006).

Our results provide evidence that *L. crispatus* slpA promotes ESC growth mainly through an ERK1/2-dependent pathway. Thus, our findings suggest that slpA might be utilized to promote ESC growth. However, further studies are necessary before clinical applications can be considered.

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References

- Babolmorad G, Emtiazi G, Emamzadeh R (2014) Analysis of the interaction between *Bacillus coagulans* and *Bacillus thuringiensis* S-layers and calcium ions by XRD, light microscopy, and FTIR. *Appl Biochem Biotechnol* 173:103–115
- Baneyx F, Mattheaei JF (2014) Self-assembled two-dimensional protein arrays in bionanotechnology: from S-layers to designed lattices. *Curr Opin Biotechnol* 28:39–45
- Bobeth M, Blecha A, Bluher A, Mertig M, Korkmaz N, Ostermann K, Rodel G, Pompe W (2011) Formation of tubes during self-assembly of bacterial surface layers. *Langmuir* 27:15102–15111
- Currie DH, Guss AM, Herring CD, Giannone RJ, Johnson CM, Lankford PK, Brown SD, Hettich RL, Lynd LR (2014) Profile of secreted hydrolases, associated proteins, and SlpA in *Thermoanaerobacterium saccharolyticum* during the degradation of hemicellulose. *Appl Environ Microbiol* 80:5001–5011
- Delhanty PJ, van der Eerden BC, van der Velde M, Gauna C, Pols HA, Jahr H, Chiba H, van der Lely AJ, van Leeuwen JP (2006) Ghrelin and unacylated ghrelin stimulate human osteoblast growth via mitogen-activated protein kinase (MAPK)/phosphoinositide 3-kinase (PI3 K) pathways in the absence of GHS-R1a. *J Endocrinol* 188:37–47
- Fagan RP, Fairweather NF (2014) Biogenesis and functions of bacterial S-layers. *Nat Rev Microbiol* 12:211–222
- Farci D, Bowler MW, Esposito F, McSweeney S, Tramontano E, Piano D (2015) Purification and characterization of DR_2577 (SlpA) a major S-layer protein from *Deinococcus radiodurans*. *Front Microbiol* 6:414
- Kitaya K, Yasuo T (2013) Regulatory role of membrane-bound form interleukin-15 on human uterine microvascular endothelial cells in circulating CD16(−) natural killer cell extravasation into human endometrium. *Biol Reprod* 89:70
- Lei L, You ZL, Fu LM, Wen LX (2006) Effects of herb therapy for benefiting qi and removing blood stasis on ultrastructure of vascular endothelial cells and vascular smooth muscle cells of uterine endometrium in rabbits with copper intrauterine device. *Zhong Xi Yi Jie He Xue Bao* 4:60–63
- Narita J, Ishida S, Okano K, Kimura S, Fukuda H, Kondo A (2006) Improvement of protein production in lactic acid bacteria using 5′-untranslated leader sequence of slpA from *Lactobacillus acidophilus*. Improvement in protein production using UTLS. *Appl Microbiol Biotechnol* 73:366–373
- Perras AK, Daum B, Ziegler C, Takahashi LK, Ahmed M, Wanner G, Klingl A, Leitinger G, Kolb-Lenz D, Gribaldo S, Auerbach A, Mora M, Probst AJ, Bellack A, Moissl-Eichinger C (2015) S-layers at second glance? Altiarchaeal grappling hooks (hami) resemble archaeal S-layer proteins in structure and sequence. *Front Microbiol* 6:543
- Quistgaard EM, Nordlund P, Low C (2012) High-resolution insights into binding of unfolded polypeptides by the PPIase chaperone SlpA. *FASEB J* 26:4003–4013
- Reus GZ, Vieira FG, Abelaira HM, Michels M, Tomaz DB, dos Santos MA, Carlessi AS, Neotti MV, Matias BI, Luz JR, Dal-Pizzol F, Quevedo J (2014) MAPK signaling correlates with the antidepressant effects of ketamine. *J Psychiatry Res* 55:15–21
- Sleytr UB, Huber C, Ilk N, Pum D, Schuster B, Egelseer EM (2007) S-layers as a tool kit for nanobiotechnological applications. *FEMS Microbiol Lett* 267:131–144
- Sleytr UB, Schuster B, Egelseer EM, Pum D (2014) S-layers: principles and applications. *FEMS Microbiol Rev* 38:823–864
- Vilen H, Hynonen U, Badelt-Lichtblau H, Ilk N, Jaaskelainen P, Torkkeli M, Palva A (2009) Surface location of individual residues of SlpA provides insight into the *Lactobacillus brevis* S-layer. *J Bacteriol* 191:3339–3349
- Xiao K, Kong F, Wang Q, Jin P, Thomas L, Xiong L, Zhou H, Gilbert GL (2013) Multiplex PCR targeting slpA: a rapid screening method to predict common *Clostridium difficile* ribotypes among fluoroquinolone resistant clinical strains. *Pathology* 45:595–599
- Yang Y, Wikel AJ, Dall’Agnol LT, Eloy P, Genet MJ, Moura JJ, Sand W, Dupont-Gillain CC, Rouxhet PG (2016) Proteins dominate in the surface layers formed on materials exposed to extracellular polymeric substances from bacterial cultures. *Biofouling* 32:95–108