molecular oral microbiology

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Involvement of lipoprotein PpiA of *Streptococcus gordonii* in evasion of phagocytosis by macrophages

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*This work is dedicated in fondest memory to Professor Takeshi Igarashi, whose influence as a mentor will be greatly missed, and without whom this work would not have been possible.

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SUMMARY

***Streptococcus gordonii* is a commensal gram-positive bacterium that resides in the human oral cavity, and is one of the most common causes of infective endocarditis (IE). Bacterial surface molecules play an important role in establishing IE, and several *S. gordonii* proteins have been implicated in binding to host cells during the establishment of IE. In this study, we identified a putative lipoprotein, peptidyl-prolyl *cis*/*trans* isomerase (PpiA), and clarified its role in evasion of phagocytosis by macrophages. Attenuation of the gene encoding prolipoprotein diacylglyceryl transferase (Lgt) altered the localization of PpiA from the cell surface to the culture supernatant, indicating that PpiA is lipid-anchored in the cell membrane by Lgt. Both human and murine macrophages showed higher phagocytic activity towards *ppiA* and *lgt* mutants than the wild-type, indicating that the presence of PpiA suppresses phagocytosis of *S. gordonii*. Human macrophages treated with dextran sulfate had significantly impaired phagocytosis of *S. gordonii*, suggesting that class A scavenger receptors in human macrophages are involved in the phagocytosis of *S. gordonii*. These results provide evidence that *S. gordonii* lipoprotein PpiA plays an important role in inhibiting phagocytic engulfment and in evasion of the host immune response.**

INTRODUCTION

Streptococcus gordonii is a commensal gram-positive bacterium that is commonly found in the human oral cavity. It plays a significant role as a pioneer colonizer in dental plaque development (Kolenbrander *et al.*, 2002). *Streptococcus gordonii* is also well known for its ability to colonize damaged heart valves, where it is frequently identified as the primary etiological agent of infective endocarditis (IE) (Baddour, 1994; Durack, 1995). Viridans group streptococci are the most common cause of native valve endocarditis in humans, accounting for 45–80% of cases (Bayliss *et al.*, 1983; van der Meer *et al.*, 1991). A variety of virulence factors have been implicated in the initial bacterial colonization of cardiac valve surfaces (Baddour *et al.*, 1989; Herzberg *et al.*, 1990; Manning *et al.*, 1994; Scheld & Sande, 1995). Based on these findings, the ability of *S. gordonii* to resist or avoid host cellular and humoral defenses

seems to be important for IE. However, the mechanism by which streptococci escape the host innate immune system during the progression of IE is not well understood.

Phagocytosis of bacterial pathogens is a crucial event in the innate immune defense of the host. Many scavenger receptors (SRs) act as pattern recognition receptors for bacterial pathogens (Mukhopadhyay *et al*., 2004; Areschoug & Gordon, 2008). Eight different classes of SRs have been proposed (A–H) according to their overall multi-domain structure (Murphy *et al*., 2005). Of these, class A SRs, including scavenger receptor A (SR-A; consisting of SR-AI, SR-AII and SR-AIII, alternate spliced forms of the same gene) and macrophage receptor with collagenous structure (MARCO), are well characterized. SR-A and MARCO are primarily expressed on phagocytes, where they act as phagocytic receptors to mediate the phagocytosis of pathogenic bacteria (Murphy *et al*., 2005), including *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Neisseria meningitidis*, *Clostridium sordellii* and *Escherichia coli* (van der Laan *et al*., 1999; Peiser *et al*., 2000; Arredouani *et al*., 2004; Mukhopadhyay *et al*., 2006; Thelen *et al*., 2010). Recent research suggests that bacterial surface proteins are major ligands for many SRs (Jeannin *et al*., 2005; Peiser *et al*., 2006; Areschoug *et al*., 2008; Plüddemann *et al*., 2009); however, the interactions between bacterial surface proteins and SRs have not been elucidated.

Bacterial surface proteins are anchored to their cell envelopes by several distinct mechanisms (Navarre & Schneewind, 1999; Cossart & Jonquieres, 2000) and play important roles in the interaction between bacteria and their environments (Sutcliffe & Russell, 1995; Navarre & Schneewind, 1999). Lipoproteins, a major group of surface proteins, have N-terminal lipid modifications, and are thought to be linked to the bacterial cell membrane. Lipoproteins were first identified in *Escherichia coli* (Hantke & Braun, 1973) and have since been identified as membrane constituents in all types of bacteria. A proposed maturation mechanism for gram-positive bacterial lipoprotein is summarized in Fig. 1. Lipoproteins are initially synthesized as precursors, with an N-terminal signal sequence, and are translocated across the cytoplasmic membrane via the Sec pathway (Sugai & Wu, 1992). The signal sequences of lipoproteins have a consensus motif called a 'lipo-box', which contains the invariable N-terminal cysteine of the mature lipoprotein. The diacylglyceryl moiety is transferred to the sulfhydryl group of the N-terminal cysteine by the prolipoprotein diacylglyceryl transferase (Lgt) (Sankaran & Wu, 1994; Qi *et al*., 1995) (Fig. 1A), followed by cleavage of the signal peptides by the lipoprotein-specific type II signal peptidase (Tokunaga *et al*., 1984; Tjalsma *et al*., 1999) (Fig. 1A) to generate mature functional lipoproteins. Lipoproteins contribute to nutrient acquisition (Perego *et al*., 1991; Russell *et al*., 1992; Alloing *et al*., 1994), adherence (Jenkinson, 1994; Kolenbrander *et al*., 1998), adaptation to environmental changes (Kappes *et al*., 1999) and protein maturation (Overweg *et al*., 2000). In addition, lipoproteins are involved in the virulence of bacterial pathogens (Stoll *et al*., 2005; Basavanna *et al*., 2009; Das *et al*., 2009; Nguyen *et al*., 2010). Hence, lipoproteins are predicted to play important roles in the interactions between pathogenic bacteria and their hosts. The lipoprotein HppA has been proposed to be an oligopeptide-binding protein (Jenkinson *et al*., 1996). However, the relationship between HppA and lipoprotein modification enzymes has not been demonstrated in *S. gordonii*. In a previous study, we confirmed that peptidyl-prolyl *cis/trans*-isomerase (PpiA) from *Streptococcus mutans* is a lipoprotein by demonstrating the link between Lgt and PpiA in surface localization. PpiA also plays an inhibitory role in the evasion of professional phagocytosis by macrophages (Mukouhara *et al*., 2011). Computational analysis of the *S. gordonii* DL1 genome database using a 'lipo-box' motif revealed that this bacterium has many putative lipoproteins. In this study, we identified a putative lipoprotein, PpiA, and examined the role of *S. gordonii* PpiA in phagocytosis by comparing the phagocytic activity of macrophages against the wild-type and *ppiA* mutant strains.

METHODS

Bacterial strains and growth conditions

Streptococcus gordonii 10558 (wild-type) and its isogenic mutants were grown anaerobically (80% N_2, 10% H_2, 10% CO_2) at 37°C in Todd–Hewitt broth medium (Difco Laboratories, Detroit, MI) (Igarashi *et al*., 2003). Strains JM109 and BL21 of *E. coli* were used as plasmid hosts and were grown aerobically either on Luria–Bertani (Invitrogen, Carlsbad, CA) agar or in Luria–Bertani broth at 37°C. When required, antibiotics were added to the medium at the

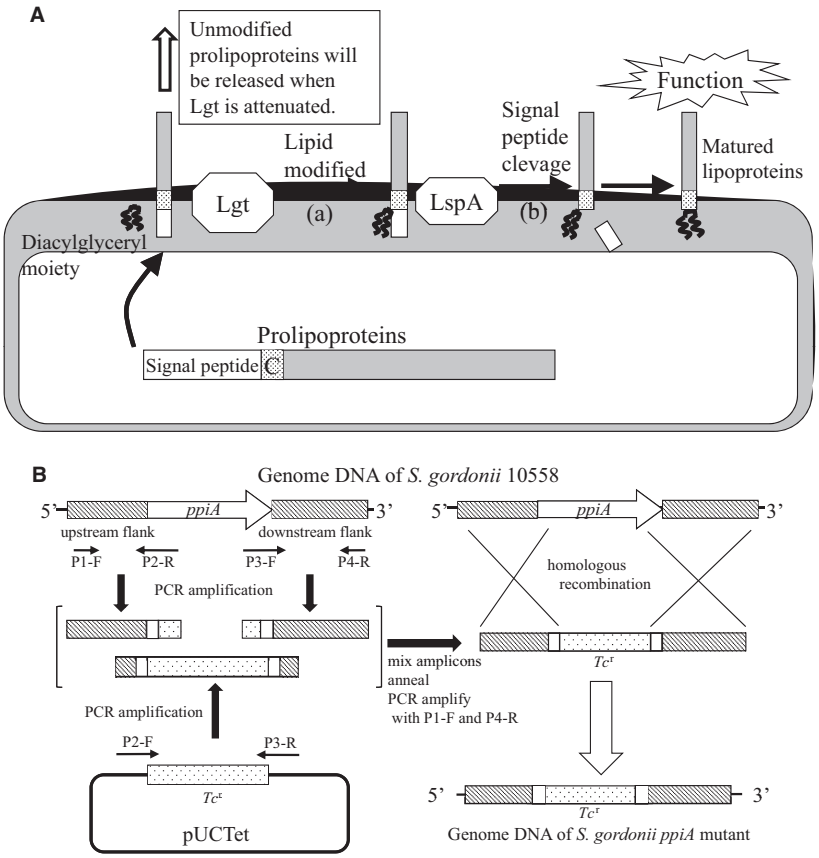

Figure 1 (A) Proposed bacterial lipoprotein maturation mechanism. A prelipoprotein is synthesized with lipoprotein-specific signal peptidase in cytosol. The prelipoprotein is transferred to the cell membrane via the Sec pathway where Lgt catalyses the lipid modification with a diacylglyceryl group (a). The lipid-modified prelipoprotein is then cleaved at signal peptide by LspA to mature lipoprotein (b). The matured lipoprotein is fixed on the cell membrane and functions. The loss of Lgt will result in a change in localization of lipoproteins from cell membrane to the culture supernatant. (B) Construction of *Streptococcus gordonii* mutants. The *S. gordonii* mutants used in this study were constructed with a vectorless PCR strategy.

following concentrations: 10 μg ml^{-1} erythromycin for *S. gordonii*; 50 μg ml^{-1} tetracycline for *S. gordonii*; and 50 μg ml^{-1} ampicillin for *E. coli*.

Cell culture

Human macrophage-like cells (designated human macrophages) were prepared as described previously (Mukouhara *et al.*, 2011). Briefly, human acute monocytic leukemia cells, THP-1 (RCB1189), purchased from the Cell Engineering Division of RIKEN Bio-Resource Center (Tsukuba, Ibaraki, Japan), were cultured in RPMI-1640 medium (Wako, Japan) supplemented with 10% heat-inactivated fetal bovine serum, 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin at 37°C in a 5% CO_2 humidified incubator. To facilitate differentiation into macrophages, THP-1 cells were cultured at an initial density of 1×10^6 cells per well on 24-well plates and treated with phorbol 12-myristate 13-acetate (PMA), at a final concentration of 10 nM, for 24 h. Following PMA treatment, the differentiated cells were washed three times with serum-free RPMI-1640 and incubated with cell culture medium. The resultant macrophage-like THP-1 cells were used for the following phagocytosis assay.

Preparation of murine peritoneal macrophages

Murine peritoneal macrophages (designated murine macrophages) were obtained as described previously (Mukouhara *et al.*, 2011) by permission of the Committee on Animal Experimentation (Showa University, Tokyo, Japan). Briefly, murine peritoneal macrophages

were accumulated by intraperitoneal injection of 1 ml 4% thioglycollate medium into 6-week-old male DDY mice (Saitama Experimental Animals Supply Co., Ltd, Saitama, Japan). At 4 days post-injection, the accumulated macrophages were harvested by peritoneal lavage with RPMI-1640 medium. The cells were plated at 1×10^6 cells per well in 24-well tissue culture plates and incubated for 2 h at 37°C in a 5% CO_2 humidified incubator, and then washed with medium to remove non-adherent cells. The adherent cells were used for the phagocytosis assay.

Phagocytosis assay

Phagocytosis of *S. gordonii* by human and murine macrophages was assessed by antibiotic protection assay, as described previously (Mukouhara *et al.*, 2011). Briefly, *S. gordonii* suspensions (2×10^6 colony-forming units) were added to the human or murine macrophages (5×10^5 cells per well) in triplicate and incubated for 45 min. After washing with phosphate-buffered saline (PBS), fresh RPMI-1640 medium (1 ml per well) containing 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin was added to kill extracellular *S. gordonii*. The infected monolayers were washed three times with PBS and lysed with distilled water to release intracellular *S. gordonii*. Serial dilutions of the lysates were plated onto Todd–Hewitt agar plates and incubated anaerobically at 37°C for 48 h. The percentage of surviving *S. gordonii* was calculated by counting recovered colony-forming units. For intracellular survival assays, after killing the extracellular bacteria, the infected macrophages were incubated for an additional 1, 3, 6 or 24 h, and then lysed with distilled water to count intracellular bacteria, as described above.

In the blocking experiment for class A SRs, human macrophages were incubated for 1 h with dextran sulfate or chondroitin sulfate before *S. gordonii* infection, as described previously (Bowdish *et al.*, 2009). The cell viability of human macrophages treated with dextran sulfate or chondroitin sulfate (each at a final concentration of 100 μg ml^{-1} or greater) was evaluated by trypan blue assay (Fardini *et al.*, 2011). Briefly, trypan blue (Invitrogen) was added to the monolayers at 1 : 10 dilution and incubated for 5 min at 37°C in a 5% CO_2 humidified incubator. The cells were then observed immediately using a Nikon TMS inverted microscope (Nikon, Japan) at 100 × magnification. Blue-stained cells were counted as non-viable.

DNA manipulation

Genomic DNA from *S. gordonii* 10558 was extracted with a GenElute bacterial genomic DNA kit (Sigma-Aldrich, St Louis, MO). Plasmid DNA was extracted using a Wizard miniprep purification kit (Promega, Madison, WI) (Igarashi *et al.*, 2003).

Construction of *S. gordonii* mutants

The ppiA-, *hppA*- and *lgt*-deficient *S. gordonii* 10558 mutants were generated by polymerase chain reaction (PCR) -based gene replacement with an antibiotic resistance gene cassette (Fig. 1B). The *S. gordonii* DL1 genome sequence (GenBank accession number CP000725) was used as a reference to design the primers. All primers and plasmids used in this study are listed in Table 1. A 294-base-pair fragment containing the upstream flanking region and upper portion of the *ppiA* gene was amplified using the P1-F/P2-R primer pair. A 261-bp fragment containing the downstream flanking region and lower portion of the *ppiA* gene was amplified using the P3-F/P4-R primer pair (Fig. 1B). The tetracycline-resistance gene (*Tc*^{r}) was amplified from pUCTet using the primer pair P2-F/P3-R. Reverse primer P2-R and forward primer P3-F were synthesized with additional nucleotides complementary to the 5′- and 3′-terminal regions of *Tc*^{r}, respectively. All three fragments were annealed in one reaction and amplified by PCR using the primer pair P1-F/P4-R. Finally, a 520-bp region of the *ppiA* gene (nucleotides 151–671) was replaced with the *Tc*^{r} gene in the resultant amplicon, which was then used to transform *S. gordonii* 10558 cells. The *ppiA*-deficient mutants were selected on Todd–Hewitt agar plates containing 50 μg ml^{-1} tetracycline. Replacement of *ppiA* with the *Tc*^{r} gene was verified by PCR. Similarly, the *lgt* gene was knocked out by replacement of the 300–513-bp region with *Tc*^{r}. The *hppA* gene was completely replaced by *Tc*^{r} by similar strategy.

RNA isolation and quantitative real-time PCR

Total RNA from human macrophages infected with wild-type *S. gordonii*, the *ppiA* mutant, or medium (uninfected control) was extracted using an RNeasy

Table 1 Oligonucleotide primers and plasmids used in this study

Primers/plasmids	Sequences/relevant characteristics[1]	Reference
Primers		
ppiAP1-F	GGTGGATCAACCTTTCTGACTTTCCC	This work
ppiAP2-F	GCAAGCAGTGCTGAAGCTAGCAAAATGAAAATTATTAATATTGGAGTT	This work
ppiAP2-R	AACTCCAATATTAATAATTTTCATTTTGCTAGCTTCAGCACTGCTTGC	This work
ppiAP3-F	CAATAAAATAACTTAGTGGCCAAGTTATAGACGGCATGGA	This work
ppiAP3-R	TCCATGCCGTCTATAACTTGGCCACTAAGTTATTTTATTG	This work
ppiAP4-R	CCCAAGGAGCAGTTTGTTGACAGT	This work
lgtP1-F	TCGGACCATTTGCTATTCGCTGGT	This work
lgtP2-F	TGGAGGACTGATTACAGGTGCTCTATGAAAATTATTAATATTGGAGTT	This work
lgtP2-R	AACTCCAATATTAATAATTTTCATAGAGCACCTGTAATCAGTCCTCCA	This work
lgtP3-F	GTACGATATATGTTCAATAAAATAACTTAGCTTTATGAATCTACTTGGAACTTGCTAGGC	This work
lgtP3-R	GCCTAGCAAGTTCCAAGTAGATTCATAAAGCTAAGTTATTTTATTGAACATATATCGTAC	This work
lgtP4-R	AAGAGAGCAGACAACCACTGGGAA	This work
hppAP1-F	TCCGTAATTCCCCCCTATCTCAA	This work
hppAP2-F	TAAATTTGAAATTGAAATGGAGAATATACGATGAAAATTATTAATATTGGAGTTTTAGCT	This work
hppAP2-R	AGCTAAAACTCCAATATTAATAATTTTCATCGTATATTCTCCATTTCAATTTCAAATTTA	This work
hppAP3-F	GTACGATATATGTTCAATAAAATAACTTAGCAAGAAGAGTTAGAAAAACACGTGAAATAA	This work
hppAP3-R	TTATTTCACGTGTTTTTCTAACTCTTCTTGCTAAGTTATTTTATTGAACATATATCGTAC	This work
hppAP4-R	CGTATCATAATAATCCACATAGCCCATACG	This work
rPpiAF	TTTTGCATGCTGTACTAGTATCCAACGTGTCTTGC	This work
rPpiAR	TTTTGTCGACTTATTTTTTAAAGTTGTAATCCTTGATCAC	This work
real 18SrRNAF	GGCGCCCCCTCGATGCTCTTAG	This work
real 18SrRNAR	GCTCGGGCCTGCTTTGAACACTCT	This work
real MARCOF	ATCCTGCTCACGGCAGGTACT	This work
real MARCOR	GCACATCTCTAGCATCTGGAGCT	This work
real SR-AF	TCCTTGCAGAGTCTGAATATGACACT	This work
real SR-AR	CCTCCTGTTGCTTTGCTGTAGATT	This work
Plasmids		
pUCTet	pUC18 carrying *Tc*^{r}	Arimoto and Igarashi (2008)
pGEX-PpiA	pGEX-4T-2 expression vector containing a partial *ppiA gene*	This work

[1]Tc^r, tetracycline resistance gene; single underline, *Sph*I; double underline, *Sal*I restriction site.

minikit (Qiagen, Germantown, MD) according to the manufacturer's instructions. Extracted RNA samples were treated with RNase-free DNase I (Qiagen) to remove contaminating genomic DNA. Total RNA (2 μg) was reverse-transcribed using a Superscript reverse transcriptase (RT) kit with oligo(dT) primers (Invitrogen) according to the manufacturer's protocol. Quantitative real-time PCR (qRT-PCR) was performed in a 1 × SYBR green master mix (Applied Biosystems, Warrington, UK) with specific primer pairs (2 ng μl^{-1}) and 0.5 ng μl^{-1} cDNA sample in a 20-μl total volume. Thermal cycler conditions included one cycle at 95°C for 90 s, followed by 40 cycles of 95°C for 15 s and at 60°C for 1 min. Non-template controls were also included to confirm the absence of primer–dimer formation. All samples were analysed in triplicate on an ABI Prism 7000 detection system (Life Technologies, Foster City, CA). The expression level of each target gene was normalized using the 18S rRNA gene as an internal control. Values are expressed as fold increases in mRNA levels relative to those for uninfected cells. All primers used for the qRT-PCR assay are listed in Table 1.

Expression and purification of *S. gordonii* PpiA

The partial *ppiA* gene (nucleotides 51–804) was amplified from the genomic DNA of *S. gordonii* 10558 using the rPpiA-F/rPpiA-R primer pair, and then digested with *Sph*I and *Sal*I restriction enzymes and ligated into the pGEX6P-1 expression vector (GE Healthcare UK Ltd., Buckinghamshire, England). The resultant plasmid was designated pGEX-PpiA. The PpiA protein was overexpressed as a GST-tagged recombinant protein from pGEX-PpiA in *E. coli* BL21, and was purified as a single band using the glutathi-

one S-transferase purification system, as recommended by the supplier (GE Healthcare) (Igarashi *et al.*, 2004). The purified recombinant PpiA protein was used as an antigen to prepare rabbit anti-PpiA serum (Igarashi *et al.*, 2003).

Western blot analysis

Streptococcus gordonii cells were treated with 20% (volume/volume) Triton X-114 to extract membrane lipoproteins (Shibata *et al.*, 1997). Briefly, *S. gordonii* strains were cultured at 37°C to an optical density at 600 nm (OD_{600}) of 0.5. The cell pellets were harvested, washed three times with PBS, and adjusted to OD_{600} = 1.0 with PBS. The adjusted cells were suspended in a 10 mM Tris–HCl buffer (pH 7.4) containing 0.15 M NaCl. The cell suspension (0.9 ml) was mixed with 0.1 ml 10% Triton X-114 working stock solution and rotated at 4°C for 24 h, followed by centrifugation at 10,000 ***g*** for 5 min at 4°C. The supernatant was transferred to a new tube and incubated at 37°C for 5 min. The sample was then centrifuged at 10,000 ***g*** for 3 min. The upper aqueous phase was again treated with Triton X-114 as described previously. Excess methanol was added to the lower phase to precipitate the lipophilic fraction, which included bacterial lipoproteins, and the mixture was incubated overnight at − 80°C. The samples were then centrifuged at 10,000 ***g*** for 5 min at 4°C. The supernatant was removed and the pellet was resuspended in a 10 mM octylglucoside solution. The resulting solution was used as the Triton X-114 extract (TX-114 extract). The bacterial culture supernatant was concentrated 50-fold with a Viva-Spin column (Sartorius, Goettingen, Germany) and mixed with sodium dodecyl sulfate–polyacrylamide gel electrophoresis buffer. The amounts of the concentrated proteins were determined by measuring the optical density at 280 nm. The TX-114 extracts from *S. gordonii* cultures of equal cell density, along with equal amounts of culture supernatant proteins, were analysed by Western blotting with rabbit anti-PpiA serum, as described previously (Igarashi *et al.*, 2003).

Small interfering RNA transfection

To introduce small interfering RNA (siRNA) into THP-1 cells, 5×10^5 THP-1 cells were incubated with 30 μl 15 pmol μl^{-1} siRNA containing 10 μl MultiFectam transfection reagent (Promega, Madison, WI) in RPMI-1640 medium for 4 h. MARCO-specific and SR-A-specific siRNA and control siRNA (product numbers: MARCO, sc-75747; SR-A, sc-44116; control, sc-37007) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

RESULTS

Cell surface anchoring of *S. gordonii* PpiA is catalysed by Lgt

Computational analysis of the *S. gordonii* DL1 genome database indicated that PpiA is a putative lipoprotein. To clarify the relationship between lipid modification enzymes, Lgt, and PpiA in *S. gordonii*, the localization of PpiA was analysed as described previously (Arimoto & Igarashi, 2008). For this purpose, we constructed *lgt*-deficient and *ppiA*-deficient mutants by partial replacement of the target genes with an antibacterial resistance cassette. The protein profiles of the TX-114 extracts, along with those of the wild-type and *lgt*-deficient and *ppiA*-deficient culture supernatants, were compared by Western blot analysis with anti-PpiA serum. Wild-type PpiA was detected in the TX-114 extract, but not in the culture supernatant (Fig. 2A,B, lane 1). PpiA was not detected in either the TX-114 extract or the culture supernatant of the *ppiA* mutant (Fig. 2A,B, lane 2), confirming that the observed immunoreactive 29-kDa protein band

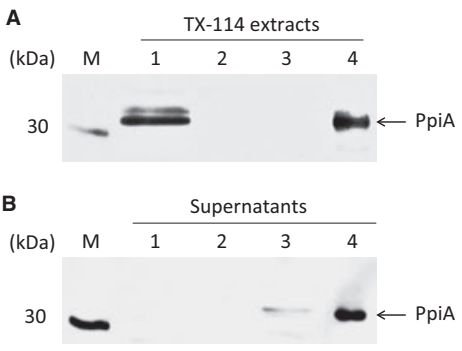

Figure 2 Western blot analysis of released and membrane-associated PpiA with rabbit anti-PpiA serum. TX-114 extracts (A) and culture supernatants (B) prepared from *Streptococcus gordonii* cells were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. PpiA (29 kDa) was detected by Western blotting with anti-PpiA serum. Lane 1, wild-type strain; lane 2, *ppiA* mutant; lane 3, *lgt* mutant; lane 4, recombinant GST-tagged PpiA protein (positive control); lane M, size marker.

was the PpiA protein, derived from the *ppiA* gene. On the other hand, in the *lgt* mutant, PpiA was found in the culture supernatant, but was not present in the TX-114 extract (Fig. 2A,B, lane 3). These results indicated that PpiA is a lipoprotein, and is anchored in the *S. gordonii* cell membrane by the Lgt enzyme.

Phagocytosis and intracellular survival of *S. gordonii* strains

To elucidate the role of *S. gordonii* PpiA in phagocytosis by macrophages, the wild-type and *ppiA*, *hppA* and *lgt* mutants were subjected to a phagocytosis assay. The number of viable *S. gordonii* cells phagocytosed by THP-1-derived human macrophages was then counted on agar plates. Compared with the wild-type, the *ppiA* and *lgt* mutants were significantly more susceptible to phagocytosis. The numbers of *ppiA* and *lgt* mutant cells phagocytosed by human macrophages were approximately 11.0- and 11.2-fold higher than that of the wild-type, respectively (Fig. 3A). The phagocytic activity of murine macrophages was similar to human macrophages: the number of *ppiA* mutant cells phagocytosed by murine macrophages was approximately 45-fold greater than that of the wild-type (Fig. 3B). The *hppA* mutant of *S. gordonii* was used to examine the involvement of lipoproteins in phagocytosis. HppA was proposed to be an oligopeptide-binding lipoprotein, and is characterized genetically and biochemically in *S. gordonii* (Jenkinson *et al*., 1996). As shown in Fig. 3A, phagocytosis of the *hppA* mutant by human macrophages was lower than the *ppiA* and *lgt* mutants, and was comparable to that of wild-type 10558. In addition, to determine whether the *ppiA* and *lgt* mutants were better able to survive in macrophages than the wild-type, intracellular survival of *S. gordonii* was examined. The result showed no differences in the intracellular survival rates of the *S. gordonii* strains tested (Fig. 3C). These results strongly suggested that the *S. gordonii* PpiA lipoprotein is specifically involved in antiphagocytic activity.

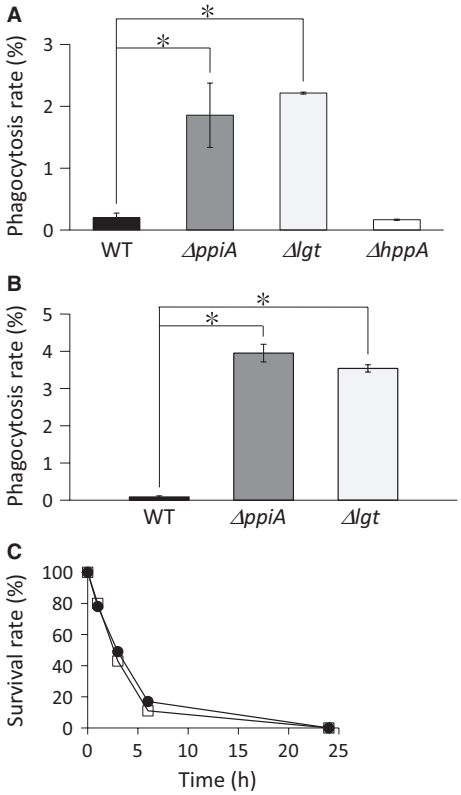

Figure 3 Phagocytosis of *Streptococcus gordonii* by macrophages. (A) Phagocytosis of the wild-type 10558 strain (WT), *ppiA* mutant, *lgt* mutant and *hppA* mutant by THP-1-derived macrophages (human macrophages). (B) Phagocytosis of the wild-type 10558, *ppiA* mutant and *lgt* mutant by murine peritoneal macrophages (murine macrophages). Data shown represent means ± standard deviations of results from three independent experiments. Phagocytosis rate is expressed as the ratio of the number of viable *S. gordonii* cells recovered to the total number of applied *S. gordonii* cells. Statistical significance ($P < 0.05$; Dunnett's test) is depicted by an asterisk. (C) The intracellular survival of *S. gordonii* in human macrophages was compared over a period of 1, 3, 6 and 24 h at postinfection. The relative survival rate was estimated by plating the lysate of infected macrophages and counting the number of colony-forming units (CFU) at each time-point. The relative survival rate is expressed as the ratio of the number of CFU at each time-point to the number of CFU at 0 h. Wild-type 10558 (●) and the *ppiA* mutant (□).

Streptococcus gordonii is phagocytosed by human macrophages via class A scavenger receptors

To determine whether class A SRs, SR-A and MARCO, are involved in phagocytosis of *S. gordonii*, the effect of an SR-A inhibitor, dextran sulfate, was examined. As shown in Fig. 4A, the number of phagocytosed cells was significantly decreased by dextran sulfate treatment. In contrast, the negative control (similar in structure) agent, chondroitin sulfate (100 μg ml^{-1}), had no effect on the phagocytosis of *S. gordonii*. Neither dextran sulfate nor chondroitin sulfate had any effect on the viability of THP-1 cells, as confirmed by trypan blue staining (data not

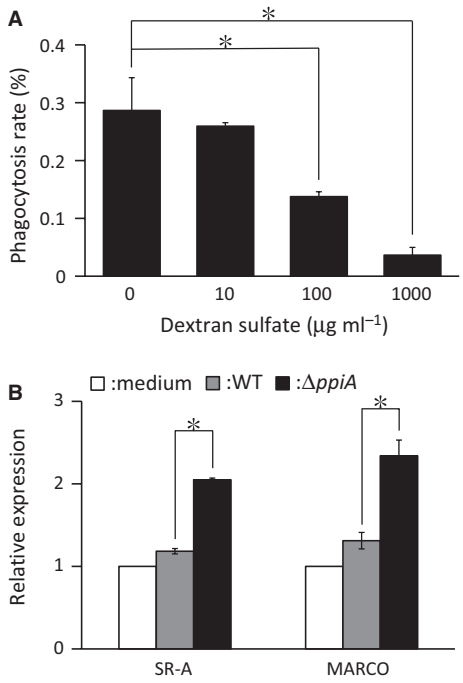

Figure 4 (A) Effect of dextran sulfate on phagocytic activities of human macrophages. Phagocytic activity of human macrophages against *Streptococcus gordonii* cells was assessed after treatment with dextran sulfate. (B) Differential transcription of class A SRs in human macrophages. Transcriptional levels of scavenger receptor A (SR-A) and macrophage receptor with collagenous structure (MARCO) genes in human macrophages stimulated with the wild-type strain and *ppiA* mutant. The relative expression levels (to unstimulated human macrophages) for SRs, corrected for 18S rRNA, are shown. Data shown represent means ± standard deviations of results from three independent experiments. Statistical significance ($P < 0.05$; Dunnett's test) is depicted by an asterisk.

shown). These results indicated that class A SRs on human macrophages play important roles in the uptake of *S. gordonii*.

Role of *S. gordonii* PpiA in phagocytosis by macrophages

Because dextran sulfate inhibited the phagocytosis of *S. gordonii* by macrophages, we focused on SR-A and MARCO. RNA from human macrophages was prepared following 45 min of stimulation by wild-type *S. gordonii* or the *ppiA* mutant, and transcriptional levels of SRs were measured by qRT-PCR (Fig. 4B). The mRNA levels of both the SRs in human macrophages stimulated by the *ppiA* mutant were twofold higher than those infected with wild-type. This result suggested that transcriptional levels of both SR-A and MARCO were upregulated by the *S. gordonii ppiA* mutant.

Effect of class A SR siRNA on *S. gordonii* uptake by macrophages

To elucidate the relationship between the decreased transcription of MARCO and SR-A in macrophages during phagocytosis of *S. gordonii*, THP-1 cells transfected with MARCO-specific or SR-A-specific siRNA were challenged in an *S. gordonii* phagocytosis assay. Endogenous expression of MARCO and SR-A was decreased by 40% and 50%, respectively, in

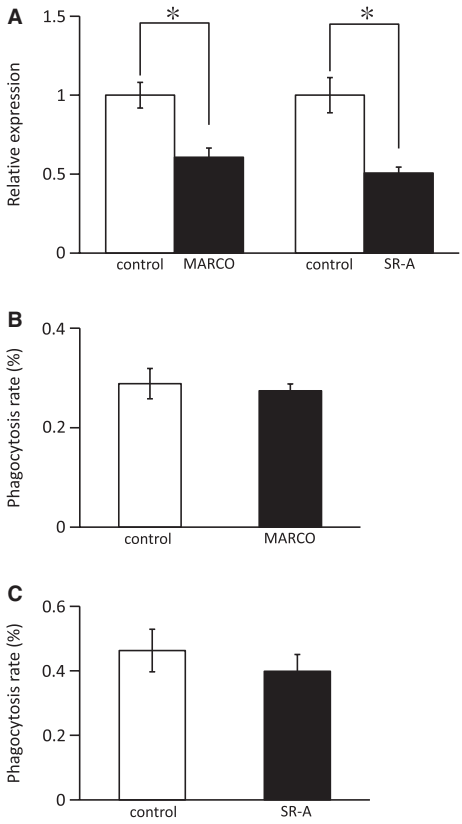

Figure 5 (A) Transfection of THP-1 cells with macrophage receptor with collagenous structure (MARCO) -specific and scavenger receptor-A (SR-A) -specific small interfering (si) RNAs. THP-1 cells were transfected with control siRNA or MARCO-specific or SR-A-specific siRNA. (B) Phagocytosis of the *Streptococcus gordonii* wild-type and *ppiA* mutant by human macrophages transfected with MARCO-specific siRNA. (C) Phagocytosis of the *S. gordonii* wild-type and *ppiA* mutant by human macrophages transfected with SR-A-specific siRNA. Data shown represent means ± standard deviations of results from three independent experiments. Statistical significance ($P < 0.05$; Dunnett's test) is depicted by an asterisk.

cells transfected with corresponding siRNA, compared with those transfected with control siRNA (Fig. 5A). There was no apparent difference in the number of phagocytosed *S. gordonii ppiA* mutants between THP-1 cells transfected with MARCO-specific or SR-A-specific siRNA and those transfected with control siRNA (Fig. 5B,C). These results suggested that decreased transcription of either MARCO or SR-A alone did not suppress *S. gordonii* uptake by human macrophages.

DISCUSSION

Surface proteins are thought to play important roles in the establishment of IE, and several surface proteins implicated in IE have been identified in *S. gordonii.* CshA, a cell wall-anchored polypeptide, mediates binding to immobilized human fibronectin, which is thought to be an important step in the pathogenesis of IE (Lowrance *et al.*, 1990; McNab *et al.*, 1996). A synergistic function for Hsa and SspA/SspB has been suggested in platelet aggregation (Jakubovics *et al.*, 2005), and recently, a novel cell wall-anchored protein, PadA, has been identified and shown to bind to the platelet fibrinogen receptor GPIIbIIIa (Petersen *et al.*, 2010). In addition, the sialic acid-binding adhesin, Hsa, and the serine-rich cell wall glycoprotein, GbpB, are important in the rat model of IE (Takahashi *et al.*, 2006; Xiong *et al.*, 2008). In the present study, we identified an *S. gordonii* virulence lipoprotein, PpiA, which is associated with evasion and survival in innate immune cells such as macrophages. PpiA was annotated as a putative lipoprotein because it contains a lipo-box motif at the C-terminus of the signal peptide. However, it has not been demonstrated experimentally in *S. gordonii* whether the PpiA lipoprotein is anchored to the cell membrane by the Lgt enzyme. We compared the localization of PpiA in wild-type *S. gordonii* as well as in the *lgt* mutant strain and found that Lgt catalyses PpiA anchoring to the cell membrane in the wild-type, implying that PpiA is modified by Lgt.

Phagocytosis assays revealed that the *S. gordonii lgt* mutant was more highly phagocytosed by macrophages than the wild-type 10558 strain, which suggests that the loss of lipoproteins from the cell surface is strongly related to increased susceptibility to phagocytosis. A similar phenomenon was also observed with the loss of the PpiA lipoprotein; however, *S. gordonii* with reduced HppA lipoprotein showed no difference in susceptibility to phagocytosis by human macrophages. These results suggest that PpiA is specifically responsible for the anti-phagocytic activity of *S. gordonii* towards human macrophages. To generalize such a possibility, susceptibility to phagocytosis of other *S. gordonii* lipoprotein mutants must be tested. In a previous study, we identified the PpiA lipoprotein of *S. mutans* as a homolog of the pneumococcal lipoprotein rotamase A (SlrA) (Mukouhara *et al.*, 2011). Similar reductions in phagocytosis have been observed for the *slrA* mutant of *S. pneumoniae* and for the *S. mutans ppiA* mutant (Hermans *et al.*, 2006; Mukouhara *et al.*, 2011). In addition to the *ppiA* genes of *S. gordonii* and *S. mutans*, a pneumococcal SlrA-homologous putative lipoprotein gene has been identified, and is closely related to those found in other *Streptococcus* species, including *S. pyogenes*, *S. agalactiae*, *S. suis*, *S. mitis* and *S. sanguinis*. Therefore, it is likely that the SlrA homologs in these bacteria are also associated with anti-phagocytic activity.

Phagocytosis assays using dextran sulfate suggested that class A SRs act as phagocytic receptors for *S. gordonii* (Fig. 4A). A similar phagocytosis assay using HL60 (human promyelocytic leukemia cells) was also performed, and revealed that neutrophils (HL60) treated with dextran sulfate were not impaired in their ability to phagocytose bacteria (data not shown). This result might support the idea that dextran sulfate blocks class A SRs on macrophages only. Because expression of class A SRs is enhanced by bacterial infection (van der Laan *et al.*, 1999; Doyle *et al.*, 2004; Mukhopadhyay *et al.*, 2004), we investigated whether the transcriptional levels of SR-A and MARCO were induced by *S. gordonii* infection. As shown in Fig. 4B, infection with the *ppiA* mutant enhanced expression of MARCO and SR-A at the transcriptional level in human macrophages, compared with those in cells that had been infected with wild-type. This finding suggested that *S. gordonii* PpiA negatively regulates expression of MARCO and SR-A, or that PpiA suppresses the up-regulation of these molecules in human macrophages (Fig. 4B). However, THP-1 cells transfected with SR-A-specific or MARCO-specific siRNA phagocytosed *S. gordonii ppiA* mutant cells at a level comparable with control siRNA, indicating that reduced transcription of SR-A or MARCO does not affect the phagocytosis of *S. gordonii*. The *S. gordonii* PpiA affected the transcription of both the MARCO and SR-A genes, whereas *S. mutans* PpiA only affects MARCO expres-

sion (Mukouhara *et al.*, 2011). It has been suggested that the role of SR-A in bacterial phagocytosis depends on the microorganism (Peiser *et al.*, 2000). It is likely that because the roles of SlrA-homologous lipoproteins are species-dependent, the contribution of SR-A to phagocytosis is also dependent on the microorganism. Until now, the molecular mechanism of the anti-phagocytic activity of the PpiA lipoprotein in *S. gordonii* has been unclear. Negative regulation of MARCO expression by *E. coli* infection has been reported (Pinheiro da Silva *et al.*, 2007). This previous study determined that binding of *E. coli* to CD16 (FcγRIII) triggers an FcγR pathway, which causes negative regulation of MARCO expression; however, interaction of *E. coli* components with CD16 has not been shown. In addition, gram-positive *Staphylococcus aureus* shows no interaction with CD16. These findings suggest that CD16-related MARCO regulation is specific to gram-negative bacteria. Hence, negative regulation of MARCO by gram-positive bacteria is not well understood. On the other hand, Toll-like receptors (TLRs) have been proposed to regulate the bacterial phagocytic activity of macrophages (Doyle *et al.*, 2004; Amiel *et al.*, 2009). It has been reported that SR-A$^{+/-}$ TLR4$^{+/-}$, but not SR-A$^{+/-}$ TLR2$^{+/-}$, bone marrow-derived dendritic cells have an impaired ability to phagocytose *E. coli*, whereas phagocytosis of *Staphylococcus aureus* is impaired in SR-A$^{+/-}$ TLR2$^{+/-}$ bone marrow-derived dendritic cells (Amiel *et al.*, 2009). As bacterial lipoproteins are major ligands for TLR2 (Akira, 2003), *S. gordonii* lipoproteins, including PpiA, seem to be candidates for the TLR2 ligand. However, we showed that the *S. gordonii lgt* mutant, in which all lipoproteins were removed from the cell surface, was more susceptible to phagocytosis than wild-type strain 10558. This suggests that recognition of lipoproteins by TLR2 is not directly related to regulation of expression of class A SRs and the subsequent bacterial phagocytosis by macrophages.

It has been suggested that lipopolysaccharide and lipoteichoic acid are recognized by both MARCO and SR-A. In addition, recent studies have suggested that bacterial surface proteins play an important role as target molecules for these receptors. For example, surface proteins from *N. meningitidis* have been shown to be ligands for MARCO (Jeannin *et al.*, 2005; Peiser *et al.*, 2006; Areschoug *et al.*, 2008; Plüddemann *et al.*, 2009), and SR-A has been shown to bind to the Blr surface lipoprotein of *S. agalactiae* (Areschoug *et al.*, 2008). From this information, we initially hypothesized that *S. gordonii* lipoproteins might be ligands for SR-A. However, this possibility seems unlikely because both the *ppiA* mutant and the *lgt* mutant were as effectively phagocytosed by macrophages as the wild-type (Fig. 3A, B). It is probable that the loss of lipoprotein PpiA changes the environment on the cell surface of the mutant, in addition to enhancing the expression of class A SRs on human macrophages. Also, such alteration might confer suitable surface conditions for easy recognition of target molecules on bacteria by MARCO and/or SR-A. Therefore, we speculate that *S. gordonii* PpiA might be involved in preventing the recognition of surface molecules (lipoteichoic acid and/or other surface proteins) by class A SRs, which negatively influences SR expression.

Our data showed that *S. gordonii* PpiA is anchored on the cell membrane by Lgt, and further indicated that loss of Lgt affects the physiological function of PpiA. Therefore, Lgt could be an attractive target molecule for therapies to prevent *S. gordonii*-related IE, though further studies should be conducted to verify this. Finally, we believe the present study is the first to demonstrate the relationship between lipoproteins and the lipid modification enzyme, Lgt, in *S. gordonii*. *Streptococcus gordonii* PpiA is lipid-anchored to the cell membrane by the Lgt enzyme, and plays an important role in anti-phagocytic activity. Therefore, we conclude that PpiA could be a useful antigen for vaccine development, and represents an attractive target molecule to develop new treatments to protect against IE, with which *S. gordonii* infection is closely associated.

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