A small GTPase-like protein fragment of Mycoplasma promotes tumor cell migration and proliferation *in vitro* via interaction with Rac1 and Stat3

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Abstract. The Mycoplasma genus comprises a group of microbes that cause persistent infection in humans and its role in promoting tumor development has long been a concern. Although mixtures of components isolated from Mycoplasma have been shown to activate host Rho family small GTPases and Stat3, no individual factor with this activity has been reported. In the current study, a conserved small GTPase-like protein fragment (SGLP) from *Mycoplasma pulmonis* chromosome partition protein, Smc, was identified as a virulence factor. SGLP was observed to interact with Rac1 and Stat3. The wild-type (wt) SGLP, which contains a WxxxE motif, induced activation of Rac1 and phosphorylation of Stat3 at the tyrosine-705 residue, while the SGLP mutant containing a mutation from WxxxE to AxxxA did not exert the same effects. Moreover, SGLP-induced Stat3 phosphorylation was observed to be dependent upon Rac1 activity. Furthermore, wt SGLP was observed to promote cell migration and increase bromodeoxyuridine incorporation in HeLa cells and the SGLP mutant did not elicit these effects in HeLa cells. In conclusion, the current observations suggest that SGLP is an important virulence factor of Mycoplasma, which contributes to tumor cell migration and proliferation *in vitro* via interaction with Rac1 and Stat3.

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Introduction

Persistent inflammatory microenvironment induced by bacteria, including Mycoplasma, is conducive to tumor development in host tissues (1). Mycoplasma promotes multistage malignant transformation of host cells following a long-term infection (2,3). Being different from other bacteria, Mycoplasma lacks a rigid cell wall, which facilitates fusion with the host cell membrane (4). Following fusion, Mycoplasma may lead to various alterations in the affected cells, which involves the Mycoplasma membrane components inserting into the host cell membrane. Among those mycoplasmal components, the lipid-associated membrane proteins have been reported to activate the Jak/Stat3 pathway through mediating host small GTPases of the Rho family, including Rac1 (5,6).

Rac1 is a significant member of Rho-family small GTPases, which are pleiotropic in controlling actin cytoskeleton reorganization (7) and Stat3 is an important transcription factor that may be activated by the GTP-bound form of active Rac1 (8-10). In terms of subcellular localization, active Rac1 contributes to Stat3 nuclear translocation by forming a complex with a Rac1/Cdc42 GTPase-activating protein (11,12), whereas cytoplasmic Stat3 regulates Rac1 activity to modulate directional cell migration (13). The functional and physical interactions between Rac1 and Stat3 potentiate cell proliferation and migration in certain cell types (8,13-15).

There are a number of bacterial virulence factors that possess a WxxxE motif, which may subvert cellular functions via interacting with host small GTPases of the Rho family. These identified factors include: The *Shigella* effectors, IpgB1 and IpgB2 (16), the *E. coli* effector, Map (17), the Salmonella effector, SifA (18) and *Pantoea stewartii subsp. stewartii* and *Pseudomonas syringae* effectors, WtsE and AvrE1, respectively (19).

Through computational analysis of Mycoplasma genomes, the chromosome partition protein Smc (Smc) conserved among Mycoplasma species was observed to possess an invariant WxxxE motif at its N terminus, which is associated with Rho family small GTPases, as well as a probable coiled

coil domain downstream of the WxxxE motif, which may interact with Stat3 as predicted by an online program designed for predicting the parallel coiled coil interaction (20). Notably, Stat3 is a binding partner of Rac1 (8); therefore, the present study aimed to determine whether the N-terminal domain of Smc interacts with Rac1 and/or Stat3.

The N-terminal sequence (amino acids 9-248) of Smc (UniProt: Q98PK8) was cloned from Mycoplasma *pulmonis*, a common Mycoplasma contaminant in cell cultures. The cloned 240-amino acid N-terminus of Smc possesses a characteristic GTP-binding motif of GxxxxGKS/T that is observed in all small GTPases. With regard to such a similarity with small GTPases, this small GTPase-like protein fragment of 240 amino acids from Smc is referred to as SGLP.

In the current study, the interaction of SGLP with Rac1 and Stat3 was investigated and the mechanism by which SGLP may be involved in affecting tumor cell migration and proliferation through interaction with Rac1 and Stat3 was demonstrated.

Materials and methods

Antibodies. The primary antibodies used were: p-Stat3 (9145) from Cell Signaling Technology, Inc. (Beverly, MA, USA); glutathione S-transferase (GST; sc-33614) and Rac1 (sc-217) from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA); FLAG (T510-2) and green fluorescent protein (GFP; T508-2) from Signalway Antibody (Baltimore, MD, USA); Myc-Tag (AM1007a) from Abgent (San Diego, CA, USA); anti-bromodeoxyuridine (BrdU; 560810) from BD Biosciences (San Jose, CA, USA) and Stat3 (51076-2-AP) from Proteintech Group (Chicago, IL, USA). Other antibodies used, including FITC-conjugated goat anti-mouse, Alexa Fluor 488-conjugated goat anti-rabbit, Alexa Fluor 594-conjugated goat anti-mouse antibodies and HRP-conjugated goat antibodies against rabbit and mouse, were purchased from Jiayuan Biotech Co. Ltd. (Wuhan, China).

Overlapping polymerase chain reaction (PCR) and plasmid construction. The point mutation for tryptophan and the mutation of WVLGE to AVLGA were generated by overlapping PCR using primers as follows: i) point mutation for tryptophan of SGLP, N-terminus 5'-ATGGATCCAAATCATTTGCAGAGCCAATTC-3' and 5'-GTTCACCCAAGACCCATTTAATGGCATC-3' and C-terminus 5'-GCCATTAAATGGGTCTTGGGTGAA CAAT-3' and 5'-CACCGAATTCCTCAATTTCAAATT CTTTTAGTTTG-3'and ii) point mutation for WxxxE to AxxxA, N-terminus 5'-GATATCAAATCATTTGCAGAGCCAAT-3' and 5'-TGTGCACCCAAGACCGCTTTAATG-3' and C-terminus 5'-AAAGCGGTCTTGGGTGCACAATC-3' and 5'-GCGAATTCCTACTCAATTTCAAATTC-3'. SGLP sequences were cloned into pGEX4T1, pEGFP-C1 and pCDF1-MCS2-EF1-Puro vectors (System Biosciences, Mountain View, CA, USA). In addition, the FLAG-tag DYKDDDDK and Kozak sequences were inserted into the pCDF1-MCS2-EF1-Puro vector. DN-Rac1 (T17N) and CA-Rac1 (Q61L) pRK5 plasmids were purchased from Addgene Inc. (Cambridge, MA, USA).

Cell culture, transfection and stable cell line establishment. HEK 293T and HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Hyclone, Logan, UT, USA) and supplemented with 10% fetal bovine serum (FBS; Hyclone) at 37˚C in a humidified 5% CO_2 atmosphere. Lentiviral packaging was conducted in HEK 293T cells using pPACK™ Packaging mix (System Biosciences) according to the manufacturer's instructions. The lentiviral titers were determined by an UltraRapid Titering kit (System Biosciences). HeLa cells were transduced with SGLP lentiviral particles with 6 μg/ml polybrene (Santa Cruz Biotechnology, Inc.) for 12 h. Transduced cells were selected with 2 μg/ml puromycin (Sigma-Aldrich, St. Louis, MO, USA) for two weeks and the stable cell lines were maintained in growth medium with 1 μg/ml puromycin. Lipofectamine™ 2000 (Invitrogen Life Technologies, Carlsbad, CA, USA) was used for transfection of plasmids and small interfering RNAs according to the manufacturer's instructions.

GST pull-down assay, immunoprecipitation and western blot analysis. GST-tagged protein expression was induced by adding 0.1 mM isopropyl-β-d-thiogalactopyranoside to BL21 *E. coli* cultures at 30˚C for 6 h. GST-tagged proteins were purified by affinity chromatography with glutathione Sepharose™ 4B beads (Amersham Biosciences, Uppsala, Sweden) according to the manufacturer's instructions. HeLa cells (~$1x10^7$) were treated with 100 ng/ml IL-6 for 24 h prior to harvesting. Equal quantities of precleared lysates (~2 mg) were incubated with sepharose beads and 10 μg GST or GST-SGLP for 2 h at 4˚C. The beads were washed three times with washing buffer (pH 7.2, 10 mM $NaPO_4$, 10 mM NaN_3, 150 mM NaCl and 0.1% Tween-20). The bait and pray proteins were then eluted from beads with elution buffer (pH 8.0, 50 mM Tris-Cl and 30 mM GSH) and subjected to western blot analysis. The active Rac1 pull-down assay was conducted according to a modified method from Teng *et al* (13). The GST-Pak1-PBD, which contains the peptide ISLPSDFEHTIHVGF (CRIB domain), was purchased from Millipore (Billerica, MA, USA) and active Rac1, as well as total Rac1, were probed separately. For immunoprecipitation, the GFP- or GFP-SGLP-transfected HeLa cell lysates (1 mg) were precleared with 25 μl protein G sepharose beads (Amersham Biosciences) and incubated with 2 μg primary antibodies at 4˚C for 2 h, followed by incubation with 25 μl protein G sepharose beads at 4˚C for 2 h. Western blot analyses were performed as previously described (21).

Confocal microscopy, fluorescence resonance energy transfer (FRET) and photobleaching analyses. Immunofluorescence images were captured using an Olympus FluoView™ FV1000 confocal microscope. HeLa cells expressing SGLP and vector control were labeled with 250 nM Mitotracker at 37˚C for 15 min. The cells were then fixed, permeabilized and stained with 50 μg/ml fluorescein isothiocyanate (FITC)-phalloidin at room temperature for 2 h followed by DAPI counterstaining. The ImageJ plugins for colocalization analysis are described and may be downloaded at http://fiji.sc/wiki/index.php/Colocalization_Analysis. The colocalization of FLAG-SGLP with Stat3 was assessed with the ImageJ plugin as described by Fay *et al* (22) and the Pearson's correlation coefficients were also calculated using

ImageJ. For FRET analysis, images were recorded in three channels for donor, acceptor and transfer, respectively. The donor and acceptor bleed-through was determined and the pseudocolor image of FRET was captured using the ImageJ plugin 'FRET and Colocalization Analyzer', as described by Hachet-Hass *et al* (23). Photobleaching of DsRed-Rac1 was achieved by continuous excitation at 561 nm with full-lamp intensity. Images of GFP-SGLP and DsRed-Rac1 were recorded at intervals of 30 sec.

Transwell migration assay. Transwell assays were conducted using Transwell migration chambers with an 8-μm pore size (Corning Inc., Acton, MA, USA) according to the manufacturer's instructions. HeLa cells (~1x10^5) suspended in 500 μl of serum-free DMEM were seeded in each chamber. The migration chamber was placed into a 24-well plate with 500 μl DMEM containing 10% FBS and incubated at 37˚C with 5% CO_2. Following 8 h, cells on the upper surface were removed with a cotton swab. The migrated cells on the lower surface were fixed and stained with 1% crystal violet. Five microscopic fields (magnification, x200) were counted per filter in three independent experiments.

BrdU incorporation by flow cytometry. HeLa cells were incubated with 50 μM BrdU at 37˚C for 30 min followed by fixation in 80% ethanol at 4˚C overnight. The cells were sequentially incubated with 2 M HCl (room temperature, 30 min) and 0.1 M $Na_2B_4O_7$ buffer (pH 8.5) for DNA denaturation. Following extensive washing, the cells were immunostained at room temperature with an anti-BrdU antibody (1:50) for 2 h and a secondary FITC-conjugated antibody (1:200) for 1 h. The cells were then resuspended in phosphate-buffered saline containing 50 μg/ml propidium iodide and 125 μg/ml RNAase, prior to flow cytometry.

Statistical analysis. Analysis of data from three independent experiments were conducted using GraphPad Prism 5.0 software (GraphPad Software, Inc., La Jolla, CA, USA) and expressed as the mean ± SD. Student's t-test was used for comparisons among groups. $P<0.05$ was considered to indicate a statistically significant difference.

Results

SGLP induces actin filament reorganization in HeLa cells. As SGLP possesses a WxxxE motif, which is required for a number of bacterial virulence factors in subverting host cellular actin cytoskeleton, the actin filament patterns in HeLa cells expressing SGLP or vector control by FITC-phalloidin staining were examined. The actin cytoskeleton was observed to be markedly altered following transduction of HeLa cells with lentiviral particles expressing SGLP, which was characterized by reduced stress fibers and increased lamellipodia and filopodia (Fig. 1). As the Rho family members of small GTPases are key regulators of stress fibers, lamellipodia and filopodia (24), this result indicated that SGLP is associated with functions of host Rho small GTPases.

SGLP interacts with host Rac1 and Stat3. The results presented in Fig. 1 revealed that SGLP, which resembles a number of bacterial virulence factors that contain a WxxxE motif, was associated with Rho-family small GTPases. One of the members of Rho small GTPases is Rac1, which is a binding partner of Stat3 (8). In addition, using computational analysis, SGLP was observed to possess a coiled-coil domain that may interact with the corresponding coiled-coil domain of Stat3 (20). Therefore, it was hypothesized that there are probable functional and/or physical interactions of SGLP with Rac1 and Stat3. Thus, a possible interaction between SGLP and Rac1 and Stat3 was investigated.

A GST pull-down assay revealed that the purified GST-SGLP protein binds to host endogenous Rac1 and Stat3 (Fig. 2A). In addition, in HeLa cells, endogenous Rac1 and Stat3 were co-immunoprecipitated with GFP-SGLP using a GFP antibody (Fig. 2B). Immunofluorescence analyses revealed that FLAG-tagged SGLP colocalized with Stat3 in the nucleus and cytoplasm (Fig. 2C). The Pearson's correlation coefficient of colocalization between SGLP and Stat3 was ~0.75 as measured by ImageJ, whereas that of the control was <0.2 in 9 cells from three independent experiments. GFP-SGLP also colocalized with DsRed-Rac1 in HeLa cells coexpressing these two proteins (Fig. 2D). Although the DsRed-Rac1 distribution was homogeneous throughout the cell, which makes it difficult to interpret for colocalization directly, the FRET between GFP-SGLP and DsRed-Rac1 revealed a significant colocalization of SGLP and Rac1 compared with the control (Fig. 2D). Furthermore, consecutive confocal images showed that the GFP-SGLP (donor) intensity increased following photobleaching of the DsRed-Rac1 (acceptor) (Fig. 2E). A linear correlation was observed between the intensity of donor recovery vs. acceptor photobleaching, indicating that GFP-SGLP interacts with DsRed-Rac1 (Fig. 2F).

WxxxE motif is required for SGLP-induced activation of Rac1 and phosphorylation of Stat3. The results in Fig. 2 revealed that SGLP interacts with Rac1 and Stat3, therefore the study focused on the effect of SGLP on the function of Rac1 and Stat3. As the WxxxE motif is required for a number of bacterial virulence factors to activate cellular Rho-family small GTPases, including Rac1 (16), it was hypothesized that SGLP may also activate Rac1 and that the WxxxE motif is crucial for this effect. Therefore the study investigated whether SGLP activates Rac1, as well as whether the WxxxE motif is responsible for the activation of Rac1. The wild-type (wt) WVLGE sequence within wt SGLP was mutated to AVLGA to generate an SGLP mutant. HeLa cell lines stably expressing wt SGLP, SGLP mutant or vector, as well as parental HeLa cells were studied. Active Rac1 levels were determined by a GST-Pak1 pull-down assay followed by western blot analysis. In the presence of the WxxxE motif, wt SGLP induced greater Rac1 activation in HeLa cells as compared with the SGLP mutant and the vector control, as well as the parental cells (Fig. 3A and C). In addition, western blot analysis revealed that wt SGLP increased the phosphorylation of Stat3 at tyrosine-705 residue (p-Stat3) in HeLa cells and that the SGLP mutant did not exhibit such a prominent effect on Stat3 phosphorylation (Fig. 3B and D).

SGLP-induced Stat3 activation is dependent upon Rac1 activity. Fig. 3 shows that wt SGLP increased cellular p-Stat3 levels, which reflects its role in the activation of Stat3. Thus,

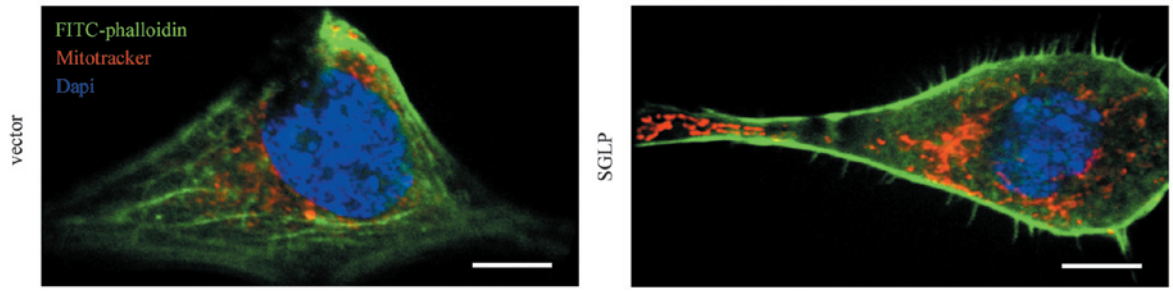

Figure 1. SGLP induces reorganization of actin cytoskeleton in HeLa cells. Confocal images of actin cytoskeleton in HeLa cells expressing SGLP or vector control. The cells were stained by fluorescein isothiocyanate-phalloidin and counterstained with mitotracker and DAPI. Scale bar, 10 μm. SGLP, small GTPase-like protein fragment.

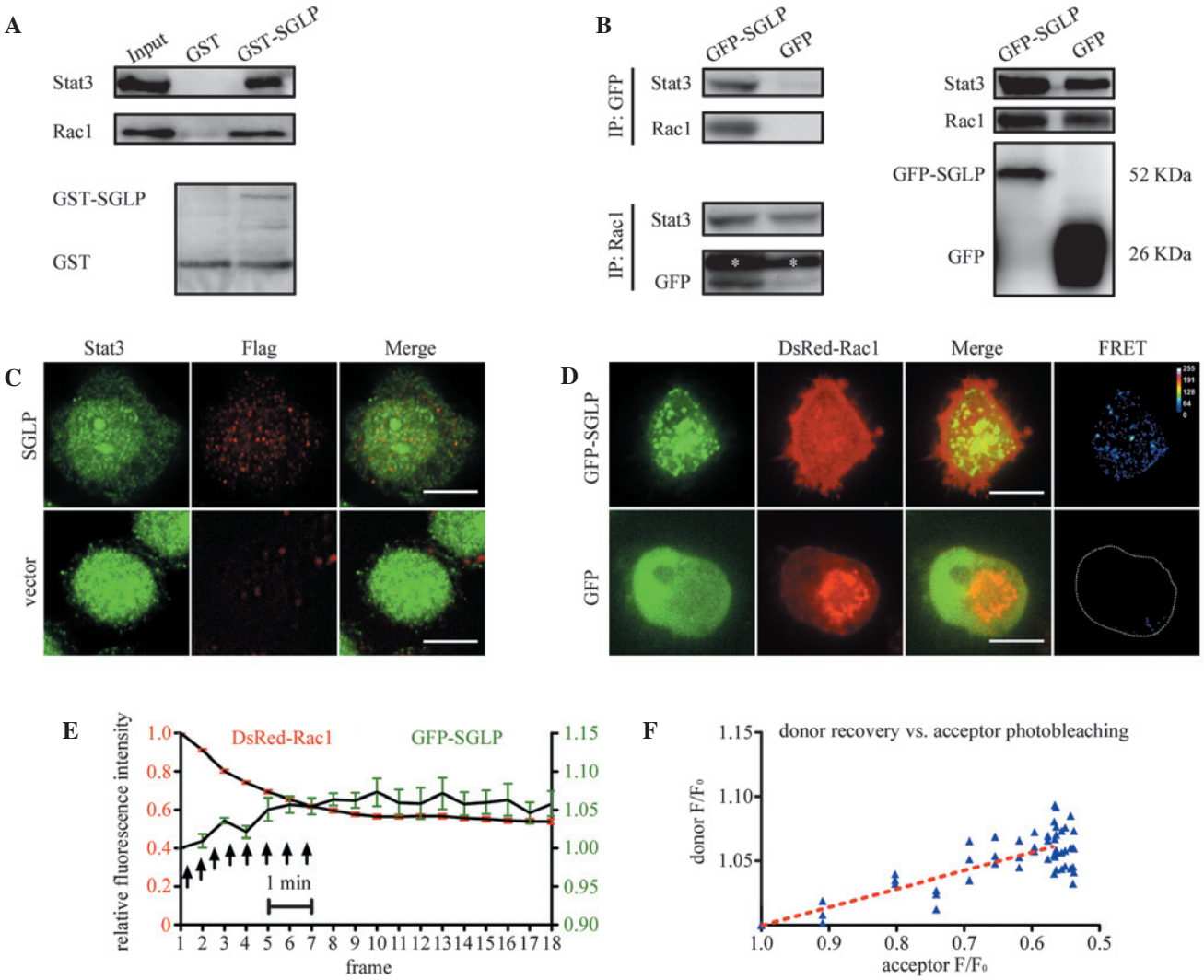

Figure 2. SGLP interacts with Rac1 and Stat3. (A) GST-tagged SGLP (~10 μg) was used to pull-down endogenous Rac1 and Stat3 from IL-6 stimulated HeLa cell lysates (2 mg). The bait protein, GST-SGLP and control GST were also immunoblotted with a GST antibody. (B) HeLa cell lysates expressing GFP-SGLP were immunoprecipitated with a GFP antibody and then probed with anti-Stat3 and anti-Rac1 antibodies. The cell lysates were also immunoprecipitated with a Rac1 antibody and then probed with anti-Stat3 and anti-GFP antibodies. The white asterisk indicates the location of the heavy chain. The right panel shows the cell lysates of input probed with indicated antibodies. (C) HeLa cells expressing FLAG-SGLP were fixed, permeabilized and immunostained with anti-Stat3 (rabbit) and anti-FLAG (mouse) antibodies in conjugation with Alexa Fluor 488 and 594 secondary antibodies, respectively. The confocal images were analyzed for colocalization using an ImageJ plugin. The boxed region is enlarged. Scale bar, 10 μm. (D) HeLa cells coexpressing GFP-SGLP and DsRed-Rac1 were fixed in 4% paraformaldehyde and observed by confocal microscopy. As DsRed-Rac1 was homogenously expressed, the FRET between GFP-SGLP and DsRed-Rac1 was measured, and the FRET indices of GFP-SGLP and GFP control were plotted as pseudocolor images. Scale bar, 10 μm. (E) HeLa cells, as in (D), were observed by confocal microscopy following photobleaching of DsRed-Rac1. Images were recorded for DsRed-Rac1 (red) and GFP-SGLP (green) every 30 sec during and following photobleaching, and the relative fluorescence intensities were plotted. The black arrows indicate the duration of full-lamp excitation. The negative GFP control did not exhibit such a recovery following photobleaching of DsRed-Rac1 (data not shown). Bar represents ± SD of nine cells from three independent experiments. (F) A plot of the linear correlation of donor recovery vs. acceptor photobleaching from (E). F_0 represents the original fluorescence intensity. SGLP, small GTPase-like protein fragment; GST, glutathione S-transferase; IL, interleukin; GFP, green fluorescent protein.

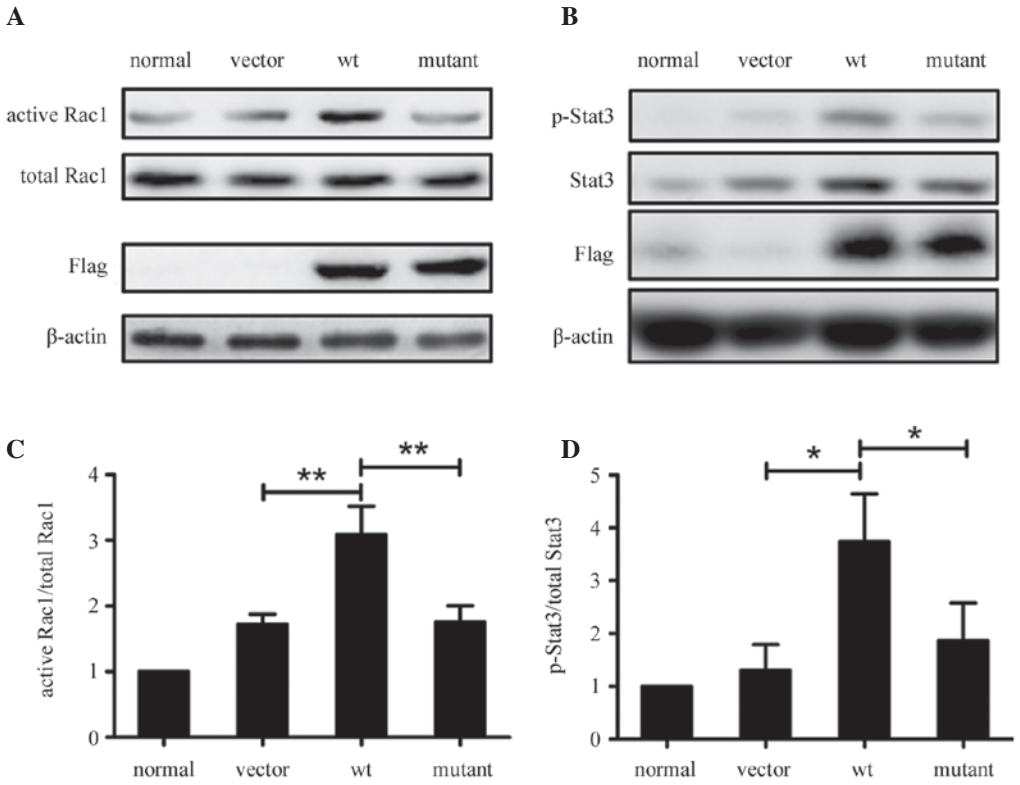

Figure 3. The WxxxE motif of SGLP is required for activation of Rac1 and Stat3. The WVLGE sequence of the wt SGLP was mutated to AVLGA to generate a SGLP mutant. HeLa cells stably expressing FLAG-tagged wt SGLP, SGLP mutant or vector control were studied in addition to the parental cells (normal). (A) A GST-Pak1 pull-down assay was used to determine the relative levels of Rac1 activation. (B) Western blot analysis of phosphorylated Stat3 (tyrosine-705; p-Stat3) and total Stat3. The densitometric ratio of (C) active-Rac1/total-Rac1 and (D) p-Stat3/total-Stat3 from three independent experiments are plotted and normalized to that of the parental cells (normal), which was set to 1. The error bar represents ± SD; t-test, n=3; *P<0.05 and **P<0.01, compared with vector and mutant groups, separately. wt, wild-type; SGLP, small GTPase-like protein fragment.

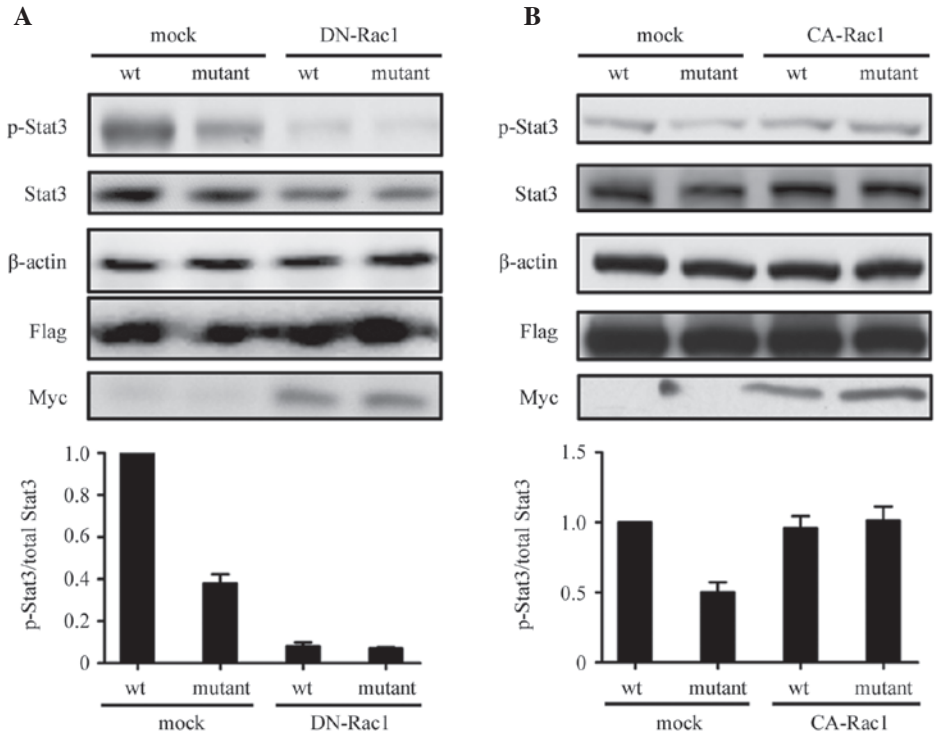

Figure 4. SGLP-induced Stat3 activation is dependent on Rac1 activity. HeLa cells stably expressing FLAG-tagged wt SGLP or SGLP mutant were transfected with either Myc-tagged (A) DN-Rac1 or (B) CA-Rac1 plasmids for 48 h and the cells transfected with p-RK5 vector plasmid were used as a control (mock). Cell lysates were probed with the indicated antibodies. In the lower panels, the densitometric ratios of p-Stat3/total-Stat3 from three independent experiments were plotted. The ratio of p-Stat3/total-Stat3 in wt SGLP-transduced HeLa cells from mock group was set to 1. The error bar represents ± SD. SGLP, small GTPase-like protein fragment; DN-Rac1, dominant negative Rac1; CA-RAC1 constitutively active Rac1; wt, wild-type.

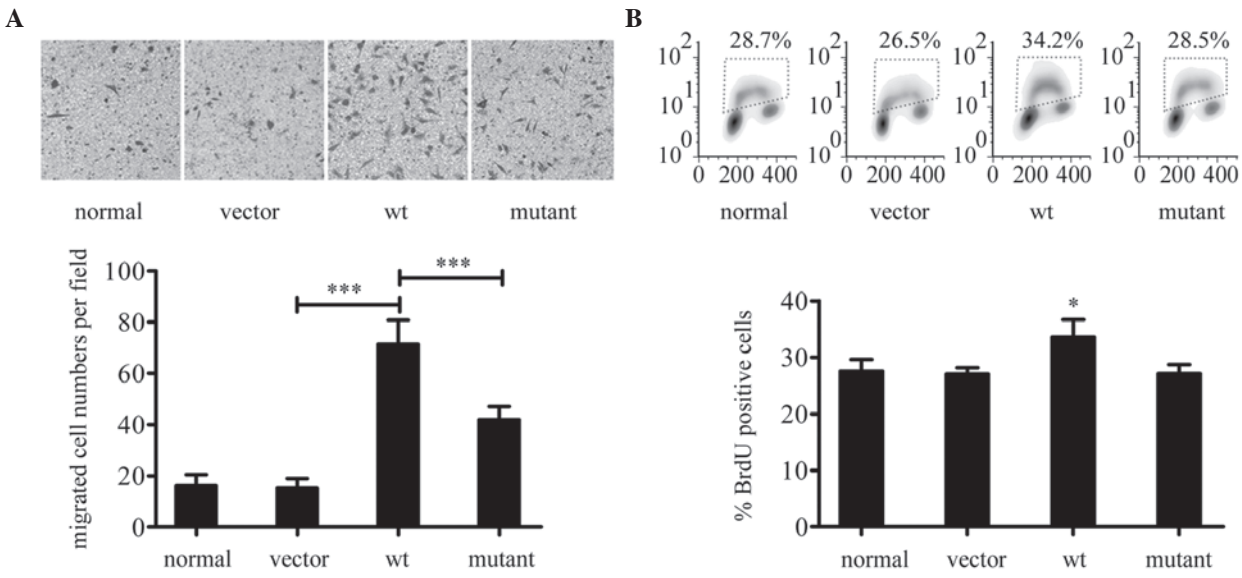

Figure 5. SGLP promotes the migration and proliferation of HeLa cells. HeLa cells stably expressing wt SGLP, SGLP mutant or vector control were observed in addition to the parental cells (normal). (A) Cells were subjected to a Transwell assay to measure cell migration. One representative result is shown (upper panel) and the average of three independent experiments is plotted (lower panel). (B) BrdU incorporation was measured by flow cytometry. The positive cell population was gated and one representative result is shown (upper panel). The average percentage of BrdU-positive cells from three independent experiments is plotted (lower panel). The error bar represents ± SD; t-test, n=3; *P<0.05, compared with other three groups, separately; ***P<0.001, compared with vector and mutant groups, separately.. wt, wild-type; SGLP, small GTPase-like protein fragment.

the mechanism by which SGLP activates Stat3 was investigated. As Rac1 mediates Stat3 activity through direct binding to Stat3 and an indirect activation loop of autocrine IL-6 (8,25), it was examined whether the SGLP-induced activation of Stat3 was dependent on Rac1 activity. The wt and mutant SGLP-transduced HeLa cells were transfected with dominant negative Rac1 (DN-Rac1) or constitutive active Rac1 (CA-Rac1) plasmids separately. Overexpression of DN-Rac1 was observed to eliminate the increase in p-Stat3 levels in wt SGLP-transduced HeLa cells (Fig. 4A), whereas overexpression of CA-Rac1 rescued the decrease in p-Stat3 levels in mutant SGLP-transduced HeLa cells (Fig. 4B). The results suggested that SGLP activates Stat3 depending on the active form of Rac1.

SGLP promotes migration and proliferation of HeLa cells. The aforementioned observations suggest that SGLP activates Rac1 and Stat3. Since Rac1 and Stat3 are involved in cell migration and proliferation (14), the possibility of whether SGLP may affect migration and/or proliferation of HeLa cells was determined. In a Transwell assay, wt SGLP was observed to increase the transwell migration of HeLa cells relative to the control and SGLP (mutant) (Fig. 5A), which is consistent with the levels of active Rac1 demonstrated in Fig. 3A. These observations suggest that the effect of wt SGLP on HeLa cell migration is associated with the wt SGLP-induced Rac1 activation. BrdU incorporation rates in HeLa cells were then investigated by flow cytometry. Wt SGLP was observed to promote BrdU incorporation (Fig. 5B), which suggests that SGLP may exert a pro-proliferative effect in HeLa cells.

Discussion

In the current study, SGLP, an N terminal protein fragment of Smc from *Mycoplasma pulmonis*, was identified as a mycoplasmal virulence factor. Homologues of SGLP may be observed in a minimum of 25 Mycoplasma species, which exhibit high levels of homology around the sequences encompassing the WVLGE motif. This conservation in evolution reflects that SGLP and its homologues may be considered as a group of common virulence factors of Mycoplasma species.

The study observed that SGLP-transduced HeLa cells exhibited reduced stress fibers and increased filopodia and lamellipodia. These phenotypic changes in actin filament distribution may have resulted from alterations in Rho-family small GTPase activity (26,27). The host Rho-family small GTPases are hypothesized to be involved in SGLP-induced cellular alterations. An interaction between SGLP and members of Rho-family small GTPases, including Rac1 was hypothesized. In addition, Stat3 directly binds to Rac1 and modulates its function (8,13). The possibility of SGLP interacting with Rac1 and/or Stat3 was investigated. SGLP was observed to form a protein complex with Rac1 and Stat3, which suggests that SGLP may interact directly with Rac1 and Stat3. SGLP was also confirmed to colocalize with Rac1 and Stat3 in the nucleus and cytoplasm, which reflects that SGLP may interact with Rac1 and Stat3 at various locales within host cells.

As SGLP is a binding partner of Rac1, the effect of SGLP on Rac1 activity was determined. SGLP was observed to increase the active Rac1 level. Since the WxxxE motif is crucial for a number of bacterial proteins to alter host Rho-family small GTPase activity (16-18), SGLP was hypothesized to cause a similar effect on Rac1 depending on the WxxxE motif. The SGLP mutant with a mutated AxxxA sequence did not induce Rac1 activation, which is consistent with the hypothesis. As active Rac1 promotes cell migration (28), it was assumed that SGLP-induced Rac1 activation may also contribute to tumor cell migration. SGLP was observed to increase cell migration

of tumor cells *in vitro*, while the SGLP mutant did not exert this effect. These results confirmed the hypothesis that the WxxxE motif is required for SGLP to increase Rac1 activity and that SGLP-induced Rac1 activation may be responsible for the observed increase in cell migration. However, this explanation does not exclude other small GTPases that may also contribute to SGLP-associated increase in tumor cell migration.

Stat3 activation is involved in a number of signaling pathways and it is pivotal in receptor tyrosine kinase-mediated pro-proliferative signal transduction (29). SGLP was observed to induce Stat3 activation. SGLP was also identified to exhibit a pro-proliferative effect on tumor cells in terms of BrdU incorporation rates, which is consistent with the observed SGLP-induced activation of Stat3. In view of the dependency of SGLP-induced Stat3 activation on Rac1 activity, it was hypothesized that SGLP may induce Rac1 activation, which, in turn, contributes to Stat3 activation. Although the role of exact domains of SGLP in forming the protein complex with Rac1 and Stat3 is somewhat unclear, the objective of the current study was to reveal and highlight a molecular mechanism by which Mycoplasma may affect host cellular responses.

In conclusion, the current findings suggest that, through interaction with Rac1 and Stat3, SGLP may potentiate tumor cell migration and proliferation *in vitro*. However, its involvement in promoting tumor development *in vivo* remains uncertain. The identification of an individual mycoplasmal virulence factor that interacts with and activates Rac1 and subsequently triggers Stat3 activation is novel. Further studies of the role of SGLP in Mycoplasma-associated pathogenesis are likely to contribute to knowledge of Mycoplasma-induced malignant transformation of mammalian cells.

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