ПРИКЛАДНАЯ БИОХИМИЯ И МИКРОБИОЛОГИЯ, 2012, том 48, № 4, с. 389–393

UDC 576.8:577.150

PUTATIVE DOWN-STREAM SIGNALING MOLECULE OF GTPase IN *Porphyromonas gingivalis*

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Department of Oral Microbiology, 2nd Stage of Brain Korea 21 for School of Dentistry, Chonnam National University, Gwangju, 500-757, Korea e-mail: shohk@chonnam.ac.kr

Received August 26, 2011

Porphyromonas gingivalis is a strict anaerobic bacterium mainly responsible for periodontal disease in oral cavity. Putative GTPase gene (*pgp*) of this bacterium was cloned and its recombinant protein (rPGP) was produced in *Escherichia coli*. Based on the amino acid sequence of SGP that is a GTP-binding protein of *Streptococcus mutans*, putative GTPase amino acid sequence was deduced in the data base of genome sequences of *Porphyromonas gingivalis*. A 900-bp PCR fragment was amplified with *P. gingivalis* genomic DNA as a template and cloned into *E. coli* JM109. Then *pgp* was transferred into pQE-30 expression vector to make pQE-PGP for production of rPGR. This protein was produced and purified by Ni-NTA affinity column chromatography. Anti-PGP antibody was also produced in Sprague Dawley rats. Using Westernblot analysis with this antibody, it was confirmed that the rPGP produced in *E. coli* was identical to that of donor strain. Furthermore, by Southernblot analysis it was revealed that the *pgp* was originated from *P. gingivalis*. By immunoprecipitation with anti-PGP antibody and N-terminal amino acid sequence analysis it was found that PGP was able to bind to acetate kinase, which was reported to be a secondary signaling-molecule in anaerobic microorganisms. Therefore, these results imply that *P. gingivalis* produces putative GTPase and this protein might play a potential role in signaling pathway in oral biofilm formation.

Porphyromonas gingivalis is one of the most well-known bacteria that cause periodontal disease. It is often found in odontogenic abscess of periodontitis patients [1]. Infection of this organism often induces the activation of osteoclastic cells resulting bone resorption [2]. Controlling this organism in oral health is important because it is associated with not only in periodontal disease but also cardiovascular disease [3]. Furthermore, the formation of oral plaque usually does not arise with single organism. Oral plaque is a microbial biofilm formed by organisms tightly bound to each other and to the solid surface of teeth by means of an exopolymer matrix into which they are embedded [4]. Organisms in biofilm compose a unique ecosystem in human oral cavity where they can successfully coexist and thrive. Characteristics of this biofilm suggest that oral bacteria may have developed some sophisticated interspecies communication mechanisms [5]. Besides that, the most of bacterial species have developed their own signaling pathways to detect environmental conditions.

GTP-binding proteins (G-proteins) are considered as ubiquitous in signaling events not only eukaryotic but also prokaryotic cells [6]. Their genes are known as essential genes for survival since several attempts to mutate this gene have been unsuccessful [7]. Several types of G-proteins were also found in prokaryotes such as *Escherichia coli* [8] and *Streptococcus mutans* [9]. Since G-protein from *S. mutans* (SGP) showed significant sequence identity with the *E. coli* Ras-like protein (Era) [8], we have searched a putative GTPase gene in *P gingivalis* based on the amino acid homology between Era and SGP. This putative gene of *P. gingivalis* G-protein (PGP) was cloned and characteristics of the purified recombinant PGP (rPGP) were investigated in this report.

MATERIALS AND METHODS

Bacteria strains. *Porphyromonas gingivalis* 381 was used as a donor strain (laboratory stock) for the cloning of GTPase gene. BHI (Brain Heart Infusion) broth (Becton Dickinson, USA) containing 0.1% vitamin K_1 (Sigma, USA) and 1% Hemin (Sigma, USA) was used for the cultivation of the bacterium. *P. gingivalis* was grown at 37°C in anaerobic chamber (Forma Scientific, USA) with 85% N_2, 10% H_2, and 5% CO_2 mixed gas. *E. coli* JM109 (Promega Co., USA) was used for the cloning of the PGP gene and *E. coli* M15 (QIAGEN, USA) was used for the expression of fusion protein.

Cloning *pgp* and expression of PGP. Putative *pgp* gene was amplified using PCR technique with *P. gingivalis* genomic DNA as a template. A SGP homologous sequence of *P. gingivalis* W83 was identified by searching The Institute for Genomic Research database (http://www.ncbi.nlm.nih.gov) with the amino acid sequence of *S. mutans*. A pair of primers, PGP-F (5'-AA GGA TCC ATG CAA GTA GAG GAG TCC-3') and PGP-R (5'-GG AAG CTT CTA ATC CAA CTG ATA GCC-3'), were designed based upon the se-

quence of *pgp* gene of *P. gingivalis* and used for amplifying and cloning.

About 0.9 kbp of PCR fragment was digested with *Bam*HI and *Hind*III and ligated into the multi cloning site of pQE-30 expression vector (QIAGEN, USA) which was also digested with *Bam*HI and *Hind*III. The resulting 4.3 kbp plasmid vector was designated as pQE-PGP and transformed into *E. coli* Ml5 which harboring pREP4 vector.

For expression of PGP protein, 5 ml of the overnight culture of *E. coli* M15 (pQE-PGP) was inoculated into 200 ml of LB broth containing 0.1 mg/ml of ampicillin and 0.025 mg/ml of kanamycin. After 2 h of the incubation at 37°C with vigorous agitation OD_{660} of the culture broth reached about 0.5. To induce the expression of PGP, 2 ml of 100 mM of isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to make 1 mM of final concentration and incubated 4 h. The culture broth was then harvested and applied to SDS-PAGE analysis and further purification steps.

Purification of recombinant PGP. Purification of rPGP was carried out by the method of manufacturer's manual with slight modifications. Briefly, the bacterial cell pellet was suspended in 10 ml of lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0) and disrupted with sonication (Vibra cell VCX-600, Sonics and Materials Inc., USA) for 5 min with a pulse (on: 5 s, off: 5 s) followed by centrifugation at 10000 *g* for 30 min at 4°C. Supernatant was saved for the following purification steps. It was loaded on 10 ml of Ni-NTA agarose resin was packed in a small column (14 × 50 mm) and the flow-through fractions were collected from the column. The column was washed with 40 ml of washing buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, pH 8.0) 3 times and the protein bound to the resin was eluted with 10 ml of elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, pH 8.0) 4 times. All the purification steps were carried out at 4°C.

Preparation of anti-PGP polyclonal antibody. Eight weeks-old female Sprague Dawley (SD) rats (180–200 g) were used for the preparation of anti-PGP polyclonal antibody. First, 0.3 mg of PGP mixed with same volume of complete Freund's adjuvant (Sigma, USA) injected in the abdominal cavity of the rats with 18-gauge needles. The same boost injection was carried out 2 weeks later. To confirm the formation of anti-PGP antibody, serum was collected from the tail of the rats and applied for the dot blot with nitrocellulose membrane (Pierce, USA). Horseradish peroxidase-conjugated goat anti-rat IgG (Pierce, USA) was used to confirm the production of antibody. After 25 days of the primary injection rats were sacrificed and serum was collected.

Immunoprecipitation. To prepare cell extract of *P. gingivalis*, bacterial culture was harvested by centrifugation at 10000 *g* for 5 min in the middle of logarithmic phase and disrupted by sonication for 5 min with a pulse (on: 5 s, off: 5 s). After centrifugation at 10000 *g* for 30 min at 4°C, the supernatant was collected. For immunoprecipitation, 0.05 ml of anti-PGP antibody and 0.1 ml of protein A-agarose beads (Sigma, USA) were added to 0.15 ml of the cell extract. The protein mixture was incubated at 4°C for 1 h with brief shaking. After centrifugation of the mixture at 10000 *g* for 15 min, the pellet was collected and washed three times with 10 mM PBS (pH 7.4) and kept at 4°C intil use.

Southernblot analysis. Partially digested chromosomal DNA with *Hind*III was applied to agarose gel electrophoresis and transferred to nitrocellulose membrane (Pierce, USA) by capillary transfer method [10]. About 0.9 kbp of *pgp* gene digested with *Bam*HI and *Hind*III from pQE-PGP was labeled with digoxigenin (Roche, Germany) to use as a probe. Probe was denatured at 95°C for 5 min, cooled rapidly on ice and hybridized to the membrane at 55°C for more than 20 h by method described above. After hybridization the membrane was washed with 2 × SSC (150 mM NaCl, 15 mM sodium citrate, pH 7.0) supplemented with 0.1% SDS twice and 0.5 × SSC with 0.1% SDS twice at 65°C (15 min for each wash). Anti-digoxigenin-AP conjugate (Roche, Germany) was diluted with 10 ml of blocking buffer (0.1 M maieic acid, 0.15 M NaCl, pH 7.5 with 0.3% Tween 20 and 1% blocking reagent with 1.0 : 5,000 ratio and applied to the membrane for 30 min at room temperature. Development of the membrane was carried out with 200 μl of NBT/BCIP (nitroblue tetrazolium/5-bromo-4-chloro-3-indoyl phosphate) solution as a substrate.

Westernblot analysis. Bacterial cell extracts and rPGP were applied to SDS-PAGE and transferred to nitrocellulose membrane. Anti-PGP antibody was used a primary antibody with 1 : 100 ratio in a blocking buffer (0.2 M Tris-HCl pH 7.6 with 1.37 M NaCl, 0.1% Tween 20 and 2% non-fat dry milk). HRP (horseradish peroxidase)-conjugated goat anti-rat IgG (Pierce, USA) was used as a secondary antibody with 1 : 2,000 ratio in the blocking buffer. Development was carried out with 0.5 mg/ml of 4-chloro-l-naphtol (Sigma, USA) and 0.05% H_2O_2.

RESULTS

Based on the amino acid sequence of a GTP-binding protein of *S. mutans* (SGP) [7, 9–12] the putative GTPase amino acid sequence was deduced in the data base of genome sequences of *P. gingivalis*. About 900-bp PCR fragment was, first, amplified with the *P. gingivalis* genomic DNA as a template and cloned into *E. coli* JM109. The structural fragment of *pgp* was, then, cut out and ligated into pQE-30 expression vector to make pQE-PGP for production of rPGP. It was transformed into *E. coli* M15 which harboring pREP4 vector. By Southernblot analysis it was confirmed that the *pgp* was originated from *P. gingivalis* (Fig. 1).

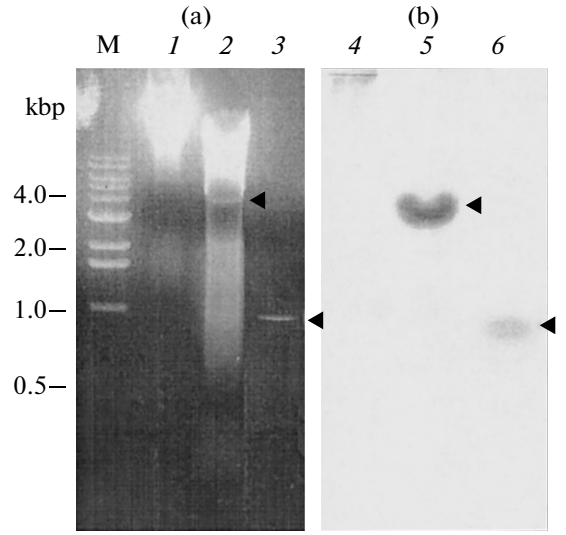

Fig. 1. Southern hybridization analysis of the *P. gingivalis* chromosomal DNA with recombinant plasmid pQE-PGP. a – chromosomal DNA from *P. gingivalis* and pQE-PGP fragments separated on 1.0% agarose gel; b – Southernblot analysis. M – molecular weight markers; *1*, *4* – chromosomal DNA digested with *Eco*RI; *2*, *5* – pQE-PGP plasmid digested with *Hind*III and *Bam*HI; *3*, *6* – 0.9 kbp-fragment of *pgp*. The position of the *pgp* is indicated by arrowheads.

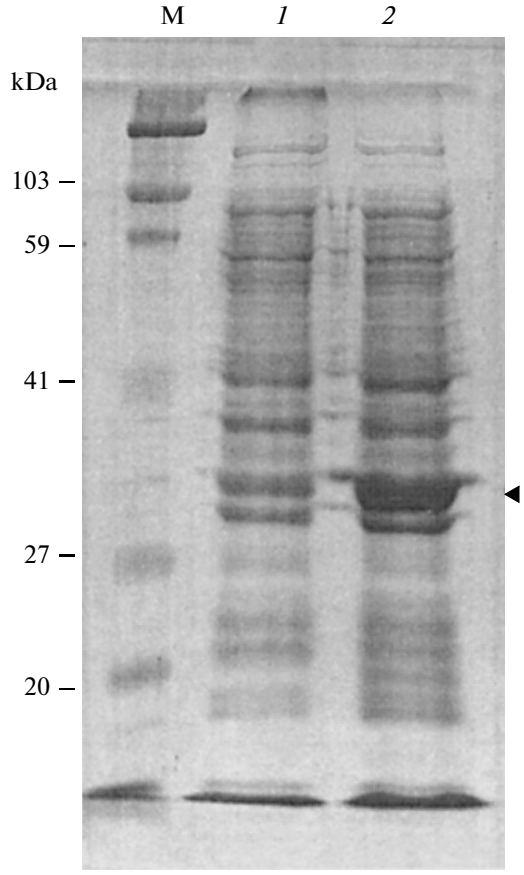

Fig. 2. Expression of PGP in *E. coli* M15. SDS-PAGE with 10% of acrylamide gel was carryied out with cell extract of *E. coli* M15 caring pQE-PGP plasmid and stained with Coomassie brilliant blue R-250. M – molecular weight markers; *1* – cell extract of *E. coli* M15 carrying pQE-PGP plasmid without induction; *2* – cell extract of *E. coli* M15 carrying pQE-PGP plasmid induced with 1 mM IPTG. rPGP expressed in *E. coli* was indicated by an arrowhead.

rPGP was produced and purified by Ni-NTA affinity column chromatography. *E. coli* M15 that harboring pQE-PGP was inoculated and cultured in LB broth. After 1 h of cultivation, 1 mM IPTG was added to induce the production of PGP (Fig. 2). Bacteria were grown for 5 h after the induction. For the purification of rPGP, the bacterial pellet was harvested with centrifuge and disrupted with sonication. Disrupted cell suspension was then applied to Ni-NTA agarose resin. After elution with imidazole with the concentration from 10 to 500 mM, rPGP was eluted at a concentration of 250 mM of imidazole. According to SDS-PAGE molecular weight of rPGP was deduced as about 33 kDa (Figs. 2 and 3).

The purified rPGP was used as an antigen to make anti-PGP antibody. Anti-PGP antibody was also produced in SD-rats. Westernblot analysis with this antibody confirmed that the rPGP produced in *E. coli* was identical to that of donor strain (Fig. 3).

With the purified rPGP, immunoprecipitation was carried out to find a molecule that might bind to PGP. After incubation of rPGP with sonic extract of *P. gingivalis*, anti-PGP antibody and protein-A-modified agarose beads, precipitate was formed. It was applied to SDS-PAGE (Fig. 4). Then the proteins transferred to PVDF membrane and one distinct protein band was cut from the membrane for N-terminal amino acid sequencing. Amino terminal sequence of the protein bound to the rPGP was turned out as acetate kinase that has a sequence of Ser-Lys-Val-Asn-Glu [3].

DISCUSSION

Putative gene of the *P. gingivalis* G-protein (PGP) was cloned into pQE-30 expression vector to make recombinant protein. Amino acid sequence of rPGP showed 91.1% with SGP (data not shown). Era which is known as an essential signal transducing molecule in *E. coli* is a peripheral membrane protein which binds to the cytoplasmic surface of the inner membrane [13]. In previous report, we have reported that there is an extensive homology between SGP and other G-proteins in the GTP-binding domain near N terminus [11]. This was also valid for PGP and SGP.

Since Era was reported to form a dimer in crystalline form [14] and SGP has been reported to oligomerize [9], it was suggested that oligomerization of

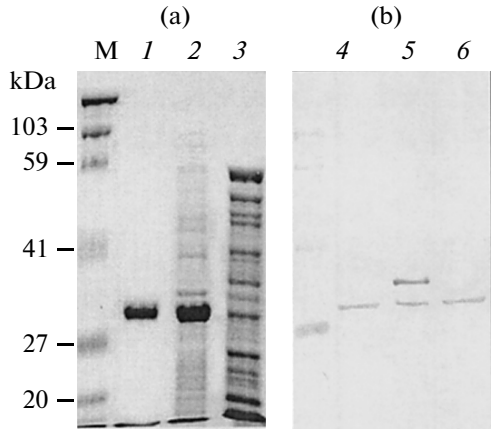

Fig. 3. SDS-PAGE and immunoblot of the purified *pgp* from *E. coli* M15 (pREP4, pQE-PGP). a – purified proteins were run on SDS-PAGE with 10% of acrylamide gels and stained with Coomassie brilliant blue R-250. b – Westernblot analysis. M – molecular weight markers; *1*, *4* – the purified rPGP; *2*, *5* – cell extract of *E. coli* M15 carrying pQE-PGP induced by IPTG; *3*, *6* – *P. gingivalis* extract.

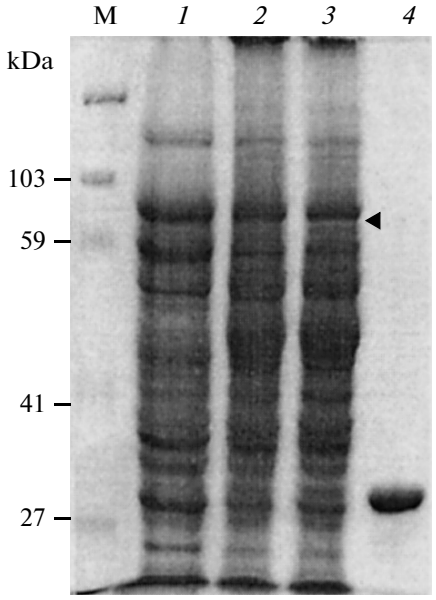

Fig. 4. Immunoprecipitation analysis of the PGP-binding protein in *P. gingivalis*. Protein extract from *P. gingivalis* was immunoprecipitated with the purified rPGP, anti-PGP polyclonal antibody and protein-A-modified agarose. M – molecular weight markers; *1* – *P. gingivalis* extract; *2* – cell extract of *P. gingivalis* immunoprecipitated with anti-PGP; *3* – cell extract of *P. gingivalis* immunoprecipitated with anti-PGP and rPGP; *4* – purified rPGP. Co-precipitated protein with anti-PGP and rPGP was indicated by an arrowhead.

G-proteins might be important for their activity. However, we could not able to find dimerized or oligomerized form of PGP in the native form. It might be due to inappropriate conditions of assay. Otherwise it might be inferred that oligomerized form of G-protein is unstable so that easily cleaved to monomer.

By immunoprecipitation with anti-PGP antibody and N-terminal amino acid sequence analysis, it was found that acetate kinase was able to bind to PGP. Acetate kinase catalyzes the reversible transfer of the γ-phosphoryl group from ATP to the carboxylate group in acetate to form acetyl phosphate. It was reported to be a secondary signaling molecule in anaerobic microorganisms playing an essential role in carbon and energy metabolism in bacteria [15, 16]. Recent report revealed that it was essential for the growth of anaerobic bacteria and anaerobic growth of *E. coli* [17].

Oral infectious diseases such as dental caries and periodontitis are caused by a series of microbial biofilms in oral cavity [18]. The formation of oral biofilm makes microbial community to have resistance to antimicrobial agents, stress and the host defense system than planktonic culture [19, 20]. Therefore, it is important to clarify the communication mechanism of intra- or extra-communications within or between species. As a candidate participating in intra-cellular signaling pathway in *P. gingivalis*, PGP might play an important role in stress-response to extracellular environment. From the results described above, it might be inferred that *P. gingivalis* produces putative GTPase and this protein might play a potential role in signaling pathway in oral biofilm formation. Further studies about this protein may provide information about the peculiar behavior mechanisms in formation of biofilm and controlling periodontal diseases [21].

ACKNOWLEDGMENTS

This study was financially supported by research fund of Chonnam National University (Korea) in 2003.

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