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Identification of a Gingipain-Sensitive Surface Ligand of *Porphyromonas gingivalis* That Induces Toll-Like Receptor 2- and 4-Independent NF-κB Activation in CHO Cells[▽]

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Porphyromonas gingivalis **is a major periodontal pathogen that has the pathogenic proteinases Arg-specific gingipain and Lys-specific gingipain. We previously found that a cell surface component on *P. gingivalis* is able to induce Toll-like receptor 2 (TLR2)- and TLR4-independent signaling in 7.19 cells and that this component can be degraded by gingipains. In this study, we purified this component from the *P. gingivalis* gingipain-null mutant KDP136 and obtained two candidate proteins. Matrix-assisted laser desorption ionization–time-of-flight mass spectrometry analysis showed that the proteins, with molecular masses of 123 and 43 kDa, were encoded by PGN_0748 and PGN_0728 (*pgm6*), respectively, in the *P. gingivalis* ATCC 33277 genome sequence. The PGN_0748-encoded protein, which we refer to as *g*ingipain-*s*ensitive *l*igand A (GslA), reacted with antiserum that could effectively inhibit the activity of KDP136 to induce NF-κB activation in 7.19 cells, but Pgm6 did not. To further determine what protein is responsible for the NF-κB activation, we constructed *gslA*, *pgm6*, and *pgm6 pgm7* deletion mutants from KDP136. When 7.19 cells were exposed to those mutants, the *gslA* deletion mutant did not induce NF-κB activation, whereas the *pgm6* and *pgm6 pgm7* deletion mutants did. Furthermore, NF-κB activation in 7.19 cells induced by KDP136 was partially inhibited by antiserum against a recombinant protein expressed from the 5′-terminal third of *gslA*. These results indicate that GslA is one of the factors that induce NF-κB activation in 7.19 cells. Interestingly, the *gslA* gene was present in four of seven *P. gingivalis* strains tested. This restricted distribution might be associated with the virulence potential of each strain.**

Porphyromonas gingivalis is an anaerobic gram-negative bacterium that is frequently isolated from advanced periodontal lesions (25). The number of *P. gingivalis* cells is closely associated with the depth of periodontal pockets and is significantly reduced after treatment (7). Thus, this organism is thought to play an important role in the development and progression of periodontitis.

P. gingivalis has two major cysteine proteinases, Arg-specific gingipain and Lys-specific gingipain. These proteinases have been reported to cleave various host immune effector molecules, such as immunoglobulin G (IgG) and IgM (22); several cytokines and cytokine receptors (1, 2, 10, 11, 17); and a pattern recognition receptor, CD14 (21, 23). These modifications of host immune regulatory molecules enable *P. gingivalis* to escape from the host immune system. This activity of gingipain seems to play an important role in the colonization of *P. gingivalis* in the oral cavity.

Besides degradation of the host molecules, we previously found that gingipains could degrade a ligand expressed on the *P. gingivalis* cell surface (8). A CHO cell-derived nuclear factor (NF)-κB reporter cell line, 7.19, was stimulated with wild-type (ATCC 33277) and gingipain-deficient *P. gingivalis* (KDP136) bacterial cells. Since bacterial cells possess a number of ligands for Toll-like receptor 2 (TLR2) and TLR4, 7.19 cells, which lack both TLR2- and TLR4-signaling pathways, enable analysis of TLR2- and TLR4-independent signaling (18). Interestingly, 7.19 cells were activated by gingipain-null mutant KDP136 but not by its parental strain ATCC 33277, suggesting that the ligand of *P. gingivalis* was degraded by gingipains in the wild-type bacterial cells. In fact, the ability of KDP136 to induce activation of NF-κB in 7.19 cells was diminished after treatment of the bacterial cells with gingipains. In a previous study (8), we partially purified components with the ability to activate NF-κB in 7.19 cells from KDP136. The activities of the components were also diminished by treatment with gingipains.

The aim of the present study was to purify and identify the gingipain-sensitive ligand from gingipain-deficient *P. gingivalis* cells. We tried further purification and obtained two proteins encoded by protein-coding sequence (CDS) PGN_0748 and CDS PGN_0728 (*pgm6*) in the *P. gingivalis* ATCC 33277 genome sequence (14). We then constructed CDS mutants from KDP136 and determined which protein is responsible for the activity that induces NF-κB activation in 7.19 cells.

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TABLE 1. Oligonucleotides used in this study

Oligonucleotide	Sequence (5′–3′)[a]
0748UPFOR	GGTATTCATTTGTCCTTGCAGAGAC
0748UPREV	CAGGGGATCCGTTAGACATCAACCACAC CCGC
0748DOFOR	TAACGGATCCCCTGACTCCTTCAAAGGCCTG
0748DOREV	GAATACGACTCTAGCCCCATGC
0728UPFOR	GCGGCCGCATCATGGGATGATCTACAGGGT
0728UPREV	GGATCCTCTCAAATATCCCCACAAATAAAT
0728DOFOR	GGATCCAATTCTGTATGTCATTTTATATTA
0728DOREV	TAATTCCATGGGTAGGTGTTGGCTACCAAC
0729DOFOR	GGATCCAGTTTTACTTTTCTAAGTGTATTT
0729DOREV	GAAGTCGCCAAAGGTGTCCTTTGCAGAACG
CEPFOR	GGATCCGACGTCAAAAGAGTTAAGGAAAGT GAAGC
CEPREV	GGATCCGACGTCTTTCAAGTCACCGATAGTG
GSLAFOR1	CATATGCCTGATCAAGAAAATAAGGAAA AGGC
GSLAREV1	GAATTCGCCTCTTCATACAAGAGTTTGACCC
GSLAFOR2	CATATGGATAAAAAGAAAAAAGAGGAGGGT ACAACG
GSLAREV2	GAATTCAGATAGGCCCTGAGGTCATCACA
GSLAFOR3	CATATGTCTGTATTTGAGCTAAATATAAGTG GCAG
GSLAREV3	GAATTCTCCTTTTTAGGATTAGGCTCCTTCCC

[a] All primers are from this study. Restriction sites incorporated into oligonucleotides for subcloning are underlined.

MATERIALS AND METHODS

Reagents. Phosphate-buffered saline (PBS), Ham's F-12 medium, penicillin-streptomycin, and trypsin-EDTA were obtained from Life Technologies (Rockville, MD). Fetal bovine serum was obtained from JRH Biosciences (Lenexa, KS). Hygromycin B was obtained from Calbiochem (San Diego, CA). DEAE Sepharose CL-6B and Superose 6 gel filtration columns were obtained from GE Healthcare (Buckinghamshire, United Kingdom). Anti-CD25 monoclonal antibody conjugated with fluorescein isothiocyanate (FITC) was obtained from Becton Dickinson (Bedford, MA). IgG1-FITC isotype control from murine myeloma was obtained from Sigma-Aldrich (St. Louis, MO). The DNA primers used in this study are summarized in Table 1.

Cell lines and flow cytometric analysis. The engineering of the CHO/CD14 reporter cell line has been previously described in detail (3). The 7.19 cell line is a CHO/CD14 cell line-derived mutant that has a point mutation at position 284 of the coding region of MD-2 that results in conversion of the codon for cysteine to tyrosine (18). Therefore, this cell line is defective in both TLR4- and TLR2-signaling pathways but can express a reporter molecule, CD25, on the cell surface through NF-κB activation induced by interleukin-1β (8). This cell line was grown as adherent monolayers in Ham's F-12 medium supplemented with 10% fetal bovine serum and hygromycin B (400 U/ml) at 37°C in a 5% CO_2 atmosphere.

For flow cytometric analysis, 7.19 cells were plated in 24-well tissue culture dishes at a density of 2.0×10^5 cells per well. After incubation for 20 h, a confluent monolayer of 7.19 cells was stimulated with freeze-dried bacteria (200 μg/ml). Following incubation for 20 h, the cells were treated with 30 mM EDTA for 1 min, and detached cells were assessed by flow cytometry for the presence of surface CD25 as described previously (8).

Bacterial strains and culture conditions. *P. gingivalis* ATCC 33277 and its isogenic mutants used in this study are shown in Table 2. The cells were grown anaerobically (10% CO_2, 10% H_2, 80% N_2) at 37°C in enriched brain-heart infusion medium supplemented with vitamin K_1 (1 μg/ml) and hemin (5 μg/ml).

TABLE 2. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
P. gingivalis strains		
ATCC 33277	Wild type	ATCC
W83	Wild type	Gift from M. J. Duncan[a]
TDC60	Wild type	Gift from K. Ishihara[b]
TDC117	Wild type	
TDC275	Wild type	
SU63	Wild type	Gift from M. Yoneda[c]
GAI-7802	Wild type	Gift from E. Hoshino[d]
KDP136	*kgp-2*::*cat rgpA2*::[*ermF ermAM*] *rgpB2*::*tetQ*	19
KDP377	*kgp-2*::*cat rgpA2*::[*ermF ermAM*] *rgpB2*::*tetQ* Δ[*gslA*]::*cepA*	This study
KDP378	*kgp-2*::*cat rgpA2*::[*ermF ermAM*] *rgpB2*::*tetQ* Δ[*pgm6*]::*cepA*	This study
KDP379	*kgp-2*::*cat rgpA2*::[*ermF ermAM*] *rgpB2*::*tetQ* Δ[λσθβ]*pgm6 pgm7*]::*cepA*	This study
E. coli strains		
XL-1 Blue	General-purpose host strain for recombinant protein production	Stratagene
BL21(DE3)	Host strain for production of recombinant proteins	20
Plasmids		
pCR4Blunt-TOPO	Ap^r Km^r; plasmid vector for cloning	Invitrogen
pBluescript II SK(+)	Ap^r; plasmid vector for cloning	Stratagene
pET22b	Ap^r; plasmid vector for cloning	Novagen[f]
pCS22	Ap^r; contains the *cepA* DNA cassette at the AatII site of pCS14	Gift from E. C. Reynolds[e]
pKD1001	Ap^r Km^r; contains 0.6-kb upstream and 0.9-kb downstream *gslA* DNA fragments in pCR4BluntII-TOPO	This study
pKD1002	Ap^r Km^r; contains the *cepA* DNA cassette at the BamHI site of pKD1001	This study
pKD1003	Ap^r; contains 0.8-kb upstream and 0.8-kb downstream *pgm6* DNA fragments in pBluescript II SK(+)	This study
pKD1004	Ap^r; contains 0.8-kb upstream and 0.8-kb downstream *pgm7* DNA fragments in pBluescript II SK(+)	This study
pKD1005	Ap^r; contains the *cepA* DNA cassette at the BamHI site of pKD1003	This study
pKD1006	Ap^r; contains the *cepA* DNA cassette at the BamHI site of pKD1004	This study
pKD1007	Ap^r; contains the 5′-terminal third of *gslA* in pET22b	This study

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[e] Cooperative Research Centre for Oral Health Science, School of Dental Science, University of Melbourne, Victoria, Australia.
[f] San Diego, CA.

For maintenance of KDP136, erythromycin (10 μg/ml) was added to the medium. For maintenance of the other antibiotic-resistant strains of *P. gingivalis*, erythromycin (10 μg/ml) and ampicillin (10 μg/ml) were added to the medium. For reporter cell stimulation experiments, the microorganisms were harvested by centrifugation, washed three times with distilled water, and freeze-dried.

Purification of the cell surface component from KDP136. The cell surface component of KDP136 was partially purified as described previously (8). Then, further purification was carried out with gel filtration chromatography (1.0-cm by 30-cm, 24-ml-column-volume Superose 6 gel filtration column) at a flow rate of 0.4 ml/min with 50 mM potassium phosphate buffer (pH 7.4) containing 0.05% CHAPS {3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate} and 0.5 M NaCl. Proteins in each fraction were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the activity of each fraction that induces NF-κB activation was determined using 7.19 reporter cells.

Protein analysis by MS. Proteins separated by SDS-PAGE were analyzed by matrix-assisted laser desorption ionization–time-of-flight mass spectrometry (MALDI-TOF MS). After in-gel tryptic digestion, peptides were extracted, concentrated, and analyzed using a Voyage-DE STR BioSpectrometry work station (Applied Biosystems, Foster City, CA). The identities of the proteins were deduced from MS peaks via the MS-Fit peptide mass fingerprinting methods in Mascot software (http://www.matrixscience.com/).

Polyclonal antibodies. To obtain antiserum against the ligand of *P. gingivalis* which can activate 7.19 cells, BALB/c mice were immunized with 30 to 250 μg of the partially purified components from KDP136 in conjunction with Freund's incomplete adjuvant. The immunization was performed at 2-week intervals, with blood being collected from the tail vein every two or three times of injection. The serum was tested for inhibitory activity to induce NF-κB activation in 7.19 cells stimulated with KDP136.

To obtain a polyclonal antibody against the PGN_0748-encoded protein, which was designated the gingipain-sensitive ligand A (GslA), rabbits were immunized with 250 μg of the recombinant protein in conjunction with Freund's incomplete adjuvant. The immunization was performed at 2-week intervals, with blood being collected every two or three times of injection. The antibody levels in the serum were measured by enzyme-linked immunosorbent assay.

Following elevation of the titer of serum antibody, animals were boosted with another immunization, which resulted in antisera. Animal care and experimental procedures were conducted in accordance with the Guidelines for Animal Experimentation of Nagasaki University and with approval of the Institutional Animal Care and Use Committee. Rabbit polyclonal antibody was purified from antiserum using protein A-conjugated Sepharose 4B (GE Healthcare).

Subcellular fractionation of *P. gingivalis*. *P. gingivalis* at mid-log phase was subjected to the following fractionation, as described previously (12). Briefly, the cells were harvested by centrifugation at 10,000 × *g* for 20 min and washed twice with 10 mM HEPES buffer (pH 7.4), suspended with 10 mM HEPES buffer (pH 7.4) that included 1 mM TLCK (*N*α-*p*-tosyl-L-lysine chloromethyl ketone) and 1 mM leupeptin, and disrupted in a French pressure cell at 100 MPa. Unbroken cells and large debris were removed by centrifugation at 1,000 × *g* for 10 min, and the supernatants (whole-cell lysates) were subjected to ultracentrifugation at 100,000 × *g* for 1 h. The precipitates were suspended in 10 mM HEPES buffer (pH 7.4) supplemented with Triton X-100 at a final concentration of 1% and mixed gently at room temperature (RT) for 30 min. The solution was subjected to ultracentrifugation at 100,000 × *g* for 1 h to yield the bacterial outer membrane fraction as the precipitate.

Construction of *P. gingivalis* mutants. To construct a *gslA* deletion mutant, two DNA fragments corresponding to the upstream and downstream regions of *gslA* were PCR amplified from the chromosomal DNA of *P. gingivalis* ATCC 33277 with two primer pairs, 0748UPFOR/0748UPREV and 0748DOFOR/0748DOREV. The two DNA fragments were annealed and subjected to PCR amplification with the primers 0748UPFOR and 0748DOREV. The resulting amplified fragment, in which *gslA* was replaced with a BamHI site, was ligated to the pCR4Blunt-TOPO vector (Invitrogen, Carlsbad, CA), resulting in pKD1001. The ampicillin resistance gene *cepA* was amplified from pCS22 with two primers, CEPFOR and CEPREV, and inserted into the BamHI site of pKD1001, resulting in a *gslA* deletion cassette, pKD1002. KDP136 was transformed to be ampicillin resistant by electroporation with linearized pKD1002 plasmid DNA to yield a *gslA* deletion mutant, KDP377 (*kgp-2*::*cat rgpA2*::[*ermF ermAM*] *rgpB2*::*tetQ gslA*::*cepA*).

To construct *pgm6* and *pgm6* PGN_0729 (*pgm7*) deletion mutants, a DNA fragments corresponding to an upstream region of *pgm6* was PCR amplified with the primers 0728UPFOR and 0728UPREV. A downstream fragment of *pgm6* was PCR amplified with the primers 0728DOFOR and 0728DOREV. A downstream fragment of *pgm7* was PCR amplified with the primers 0729DOFOR and 0729DOREV. Each resulting amplified fragment was ligated to the pCR4Blunt-TOPO vector. The vector containing the upstream fragment of *pgm6* was digested with NotI and BamHI, and the other vectors were digested with BamHI and EcoRI. The NotI-BamHI fragment, BamHI-EcoRI fragment, and pBluescript SK(−) vector (Stratagene, La Jolla, CA) digested with NotI and EcoRI were ligated, resulting in pKD1003 (target, *pgm6*) and pKD1004 (target, *pgm6 pgm7*). The amplified *cepA* gene was inserted into the BamHI site of pKD1003 or pKD1004, resulting in a *pgm6* deletion cassette, pKD1005, or a *pgm6 pgm7* deletion cassette, pKD1006. Each *cepA*-containing plasmid was subjected to transformation of KDP136 to yield a *pgm6* deletion mutant, KDP378, or a *pgm6 pgm7* deletion mutant, KDP379.

Recombinant protein. Recombinant GslA protein was prepared as described previously (15). The DNA fragment coding for the 5′-terminal 510 amino acids was amplified from *gslA* of *P. gingivalis* ATCC 33277 with two primers, GSLAFOR1 and GSLAREV1. The resulting fragments were then inserted into the NdeI-EcoRI site of plasmid pET22b to yield pKD1007. *E. coli* BL21(DE3) cells harboring the resulting plasmids were grown in LB broth. Then, isopropyl β-D-thiogalactoside was added to the culture at 0.1 μM, followed by incubation for 2 h, to overproduce the recombinant protein. The induced recombinant protein was purified using a Ni-nitrilotriacetic acid purification system (Invitrogen).

Western blot analyses. The samples were dissolved in Laemmli sample buffer and separated by SDS-PAGE. The gels were stained with Coomassie brilliant blue (CBB) or transblotted onto Immun-Blot polyvinylidene difluoride membranes (Bio-Rad Laboratories, Hercules, CA). Each membrane was blocked with 5% skim milk in PBS for 2 h at RT and reacted with the mouse polyclonal antibody against the Triton X-114-extracted bacterial cell surface components or the rabbit polyclonal antibody against the recombinant protein from the 5′-terminal third of *gslA* in 5% skim milk in PBS for 2 h at RT. Each membrane was then washed with PBS containing 0.05% Tween 20 and subjected to immunodetection using peroxidase-conjugated goat anti-mouse Ig or peroxidase-conjugated swine anti-rabbit Ig and an ECL Plus detection system (GE Healthcare).

Southern hybridization analyses. A DNA probe for the upstream region of *gslA* was prepared with PCR amplification from ATCC 33277 chromosomal DNA using the primer pair 0748UPFOR and 0748UPREV. A DNA probe for *cepA* was prepared with PCR amplification from pCS22 plasmid DNA using CEPFOR and CEPREV. DNA probes a, b, and c for internal regions of *gslA* were prepared with PCR amplification from ATCC 33277 chromosomal DNA with the three primer pairs GSLAFOR1 and GSLAREV1, GSLAFOR2 and GSLAREV2, and GSLAFOR3 and GSLAREV3, respectively. These probes were labeled with the Alkphos Direct system for chemiluminescence (GE Healthcare). Chromosomal DNA was digested with BamHI or PstI and electrophoresed in an 0.8% agarose gel. Southern blotting was performed using a Hybond-N+ membrane (GE Healthcare) and developed with CDP-Star detection reagent (GE Healthcare).

RESULTS

Purification and identification of the protein from KDP136 that induces NF-κB activation in 7.19 reporter cells. The bacterial cell surface components of *P. gingivalis* KDP136 extracted with Triton X-114 were subjected to ion-exchange chromatography as described previously (8). They were further purified by gel filtration chromatography, and the resulting fractions were analyzed for NF-κB activation-inducing activity in 7.19 cells. The fractions with high activity were subjected to SDS-PAGE, and a 43-kDa protein and a 123-kDa protein were detected (Fig. 1A). The 123-kDa protein was found in the Triton X-114-extracted bacterial cell surface components of KDP136 but not in those of *P. gingivalis* ATCC 33277.

MALDI-TOF MS analysis revealed the 123-kDa and the 43-kDa proteins as proteins encoded by PGN_0748 and PGN_0728 (*pgm6*), respectively. The PGN_0748 protein consists of 1,530 amino acids, and the peptides of the 123-kDa protein obtained by MALDI-TOF MS analysis were matched to the amino acid sequence in the region from A^{38} to R^{1219} of PGN_0748, indicating that the 123-kDa protein was encoded by at least the 5′-terminal three-quarters of the PGN_0748

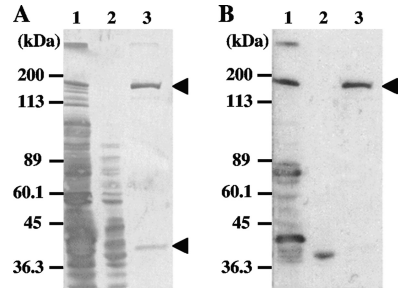

FIG. 1. Results of SDS-PAGE and Western blot analyses of bacterial components. Cell surface components of *P. gingivalis* were extracted with Triton X-114 (lanes 1, gingipain-null mutant KDP136; lanes 2, wild-type ATCC 33277). Then, the cell surface components of KDP136 were purified by ion-exchange chromatography and gel filtration chromatography as described in Materials and Methods (lanes 3). The samples were loaded onto 15% SDS-PAGE gels, and the gels were stained with CBB (A) or subjected to Western blot analysis using mouse antiserum that could effectively inhibit NF-κB activation in 7.19 cells stimulated with KDP136 (B).

gene without the putative signal peptide-encoding region (Fig. 2).

Western blot analyses were performed using mouse antiserum that was obtained by immunization with the Triton X-114-extracted bacterial cell surface components and could effectively inhibit NF-κB activation in 7.19 cells stimulated with KDP136. The antiserum reacted to the 123-kDa protein but not to the 43-kDa protein (Fig. 1B). The antiserum also reacted to a protein with a molecular mass of 40 kDa, shown in Fig. 1B, lane 2 (ATCC 33277). Since the 40-kDa protein seems not to be present in the cell surface components extracted from KDP136, the protein may be a protein product degraded from the 123-kDa protein. These results suggested that the 123-kDa protein, which we refer to as *g*ingipain-*s*ensitive *l*igand A (GslA), was a principle candidate for the ligand that induces NF-κB activation in 7.19 cells.

Responses of 7.19 cells to *gslA*, *pgm6*, and *pgm6 pgm7* deletion mutant cells. To determine which protein is responsible

1	MPDQENKEKA	LSALVDKLLR	IVKDKESKST	AEKNEWRARP	LAYETLKNLV	APNLKENERL	MKGDLLLAKE
71	CLEMSLPGSN	HLGVLKSLMT	LQSVVKLLKL	SVRREFRPND	SLIPIKGISR	VMQARLRERL	NVYDVSTLLE
141	RGCTYERRVS	MAKELGADIK	LVTSWVRQAD	LWRVDDIGSD	LAYLLVQAGV	RNVGDLAKVD	PEKAYPILYN
211	INSTQAGFTF	RGKEELERVI	RDAALLVRFS	PSTTSSYRKW	VREYEGNIRK	IAPSVINDPK	PADALLARLR
281	EDLRRIGLPR	IVGDDPTAKG	DGFPLIPAQV	EADGLPPVYL	FKDGVSLDDD	EAMIELQSIY	DILNEALGFL
351	DNIEYTLPLP	RTASGTVFIK	NANDLDENKR	ALPGVMVEID	GIVSPDQDKT	EVNKKPRCYT	DGSGKFIISM
421	PDRYSLKEAI	TIIVSDGAKK	QKFLMTASDF	INSVPEQREL	DEFLALDALG	DRADSLSERI	NYLEKKWARL
491	DKKKKEEGTT	ERVKLLYEEA	QNKISETLKG	KGDNRGLNGE	LEDLKAEYKK	KREQLLKHAP	SSDLKSAFGR
561	FMASASMLNA	KLAPSVIGDE	IKKEGEDVKS	GEVKVKSEGF	VVIQEIFERR	RMDIPRALPS	VKLMGEGSDI
631	IKLPTDTAPS	RVFTYKMLQR	LKEPDIFPVP	SGAGRNGRIP	VNRPLDVEAF	KEQMYKNPHD	YPQMSTLGIG
701	YTLNMHQAWV	PDGFALGTLL	YSLVLAPGEE	QRLIVRENKQ	RYSLADISQG	TDATRSRYAM	SQVDDSHAIF
771	QYAVDQMSKA	ESSSGYSTST	GNFGGGLGIA	GGFLPYVSAT	LGLSGGSSRA	RGRSFSSSSQ	SNSYREASMA
841	ANSFQHSIKS	ASEKLSQSRR	VSISTATSDV	SDSVATKIIA	NHNHSHAMTV	QYWEVMRRYR	LETCIDSVDL
911	VLFVPLRPIR	FLPEGQELIY	PVNNISSFGR	EEFKRRYATV	LKHADSLRYY	LPYKYRAGLD	LIQKYAALPQ
981	WTMEKLGSAP	SVFELNISGR	FLSCDDLRAY	LVLKNGKGTV	AGTVSYRRIA	LKDHYQTSRE	LKRVIRDIRN
1051	SNTFEYGESV	AKISFFLPIG	VVNEDISHVT	IRYSCEPLEY	TLYKDPDAKT	MKGESAHSEF	SKMMDKYWDL
1121	MKDNDNSSGD	LRKIEYYKKV	LPEAYISPNV	EISPGEMRSL	GVPEIRLSSA	SVGYQLVLSS	TYLDGDVYVG
1191	VSTSAHTMLY	TEFRQIEALL	QHLASETLRY	SQIVWRGLSD	DERAMMLEQY	TVKMDFEEAI	QNASLPEDEE
1261	EMIKGLDRKN	INVPLLNCVN	VKKLLGFYGN	CMLLPFTFPQ	SLSKMLGCTA	ADLQDAICRY	HSNSFRAPTT
1331	TISLPTDGMV	GEAVLGETNV	SEKIDLTRFW	NWQDSPIDKM	NIDEKSLNST	DYLVGKTTKD	ITPLNLQGPT
1401	PATPVSTVDL	LTALVNKQAP	TFDNMTGLDQ	LKEILNEATK	SAATGRDKAI	EASENMAKAA	MDFFSIGKKA
1471	EGGKEGGAPT	PKESGGENNG	GAINNVFVYP	GGTCVTGEPS	VAKPKEPDPK	EGKEPNPKKE	

FIG. 2. Results of MALDI-TOF MS analysis of the 123-kDa protein. The predicted peptides that matched to GslA are shown with underlines.

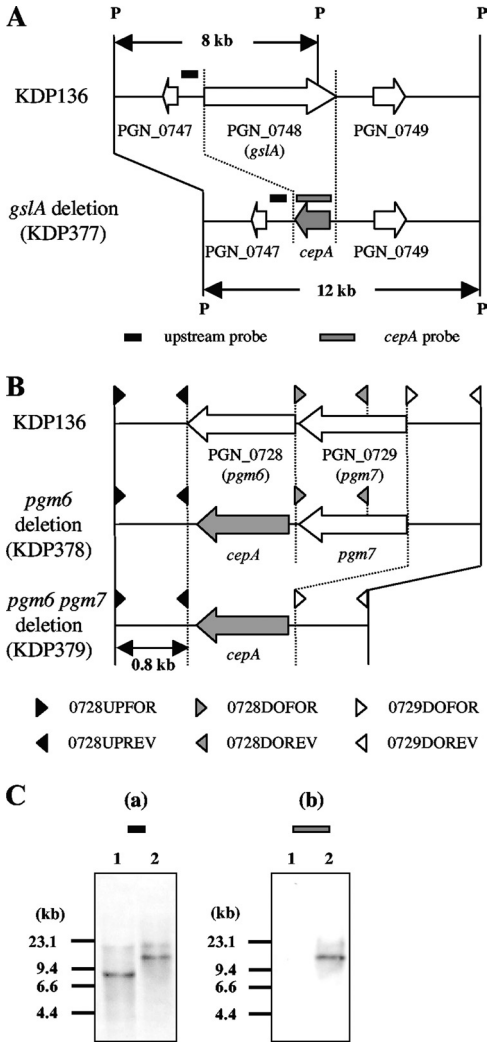

FIG. 3. (A and B) Construction of *gslA*, *pgm6*, and *pgm6 pgm7* deletion mutants. *gslA* (A) and *pgm6* alone or *pgm6 pgm7* (B) were replaced with *cepA* genes as described in Materials and Methods. Arrows show CDSs. PGN numbers are CDS numbers of *P. gingivalis* ATCC 33277. Hybridization probes for the upstream region of *gslA* (black box) and for *cepA* (gray box) are indicated (A). P, restriction enzyme-recognized site for PstI. (C) Chromosomal DNA from gingipain-null mutant KDP136 and its *gslA* deletion mutant KDP377 was digested with PstI and subjected to Southern hybridization using a probe specific for the upstream region of *gslA* (a) and a probe specific for *cepA* (b). Lanes: 1, KDP136 (*kgp rgpA rgpB*); 2, KDP377 (*kgp rgpA rgpB gslA*::*cepA*).

for the induction of NF-κB activation in 7.19 cells, the GslA-encoding gene (*gslA*) and *pgm6* and *pgm6 pgm7* deletion mutants (KDP377, KDP378, and KDP379, respectively) were constructed from the gingipain-null mutant KDP136 (*rgpA rgpB kgp*). Since proteins encoded by *pgm6* and *pgm7* form stable heterotrimers (13), we constructed the *pgm6 pgm7* mutant (KDP379) to exclude the possibility that the function of those heterotrimers was replaced by the homotrimer of Pgm7. Each target gene was replaced by *cepA*, an ampicillin resistance gene. The designs for construction of the mutants are shown in Fig. 3A and B. Correct construction of the mutants was re-

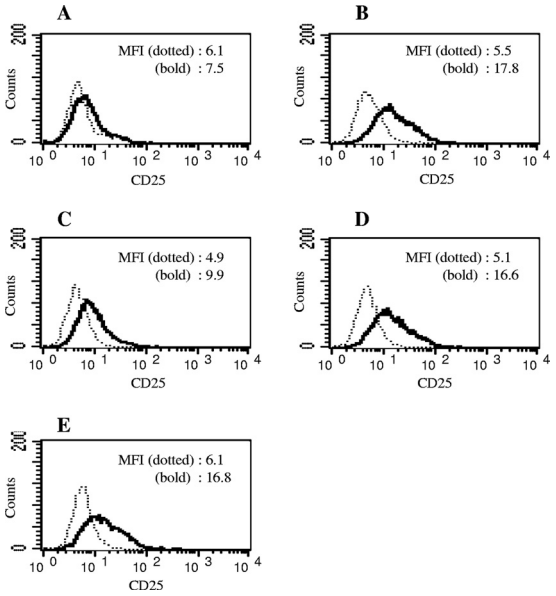

FIG. 4. NF-κB activation in 7.19 cells exposed to wild-type or mutant *P. gingivalis*. 7.19 cells were stimulated with 200 μg/ml of freeze-dried wild-type *P. gingivalis* ATCC 33277 (A), gingipain-null mutant KDP136 (*kgp rgpA rgpB*) (B), *gslA* deletion mutant KDP377 (*kgp rgpA rgpB gslA*::*cepA*) (C), *pgm6* deletion mutant KDP378 (*kgp rgpA rgpB pgm6*::*cepA*) (D), and *pgm6 pgm7* deletion mutant KDP379 (*kgp rgpA rgpB* Δ[*pgm6 pgm7*]::*cepA*) (E). Following 20 h of incubation, the cells were stained with FITC-labeled anti-CD25 monoclonal antibody (bold line) or isotype-matched control monoclonal antibody (dotted line) and subjected to flow cytometric analysis for NF-κB-driven CD25 expression. Representative results of one of three experiments performed are shown. MFI, mean fluorescence intensity.

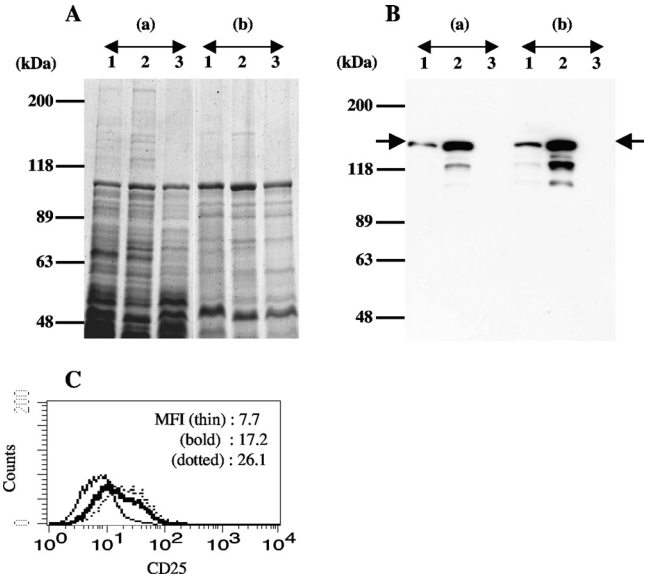

FIG. 5. Inhibition of KDP136-induced NF-κB activation in 7.19 cells by an antibody against the recombinant GslA protein. Whole-cell lysates and outer membrane fractions of *P. gingivalis* were subjected to 7% SDS-PAGE and stained with CBB (A) or reacted with the polyclonal antibody against the recombinant protein encoded by the 5′-terminal third of *gslA* (B). (a) Whole-cell lysates. (b) Outer membrane fraction. Lanes: 1, wild-type *P. gingivalis* ATCC 33277; 2, gingipain-null mutant KDP136 (*kgp rgpA rgpB*); 3, *gslA* deletion mutant KDP377 (*kgp rgpA rgpB gslA*::*cepA*). Arrows indicate the 123-kDa protein. (C) Following 20 h of incubation, 7.19 cells were stained with FITC-labeled anti-CD25 monoclonal antibody and subjected to flow cytometric analysis for NF-κB-driven CD25 expression. 7.19 cells remained unstimulated (thin line) or were stimulated with 200 μg/ml of freeze-dried KDP136 bacterial cells in the presence of 100 μg/ml of the polyclonal antibody used for Western blot analysis (bold line) or the IgG fraction from serum of a preimmunized rabbit (dotted line). Representative results of one of three experiments performed are shown. MFI, mean fluorescence intensity.

vealed by Southern blot hybridization and PCR analyses (Fig. 3C and data not shown).

7.19 cells were stimulated with wild-type or mutant *P. gingivalis* cells and subjected to flow cytometric analysis. Freeze-dried KDP136 cells induced a considerable level of NF-κB activation in 7.19 cells, whereas ATCC 33277 induced only a marginal level of activation (Fig. 4A and B). Cells of strain KDP377 showed a much lower level of NF-κB activation than did cells of strain KDP136 (Fig. 4C). Cells of strains KDP378 and KDP379 induced the same level of CD25 expression as was induced by cells of strain KDP136 (Fig. 4D and E). These results indicated that GslA was essential for the activation of NF-κB in 7.19 cells.

Western blot and inhibition assay with polyclonal antibody against recombinant GslA protein. A polyclonal antibody against the recombinant protein encoded by the 5′-terminal third of *gslA* was raised, and Western blot analysis was performed using this antibody. The antibody reacted to the 123-kDa protein in the whole-cell lysates of ATCC 33277 and KDP136, but there were no protein bands in the lysate of KDP377. In addition, the lysate of KDP136 contained a much larger amount of the 123-kDa protein than did the lysate of ATCC 33277. Similar results were obtained when outer membrane fractions were used. These results confirmed the identity of the 123-kDa protein and *gslA* product (Fig. 5A and B). Next, an experiment was performed to determine whether this antibody could inhibit the activation of NF-κB in 7.19 cells induced by freeze-dried KDP136. The activation of NF-κB in 7.19 cells was partially inhibited by this antibody compared to the level of activation induced in the presence of the IgG fraction purified from the serum of a preimmunized rabbit (Fig. 5C). Together with the results of the genetic experiments, these results indicated that GslA was responsible for the activation of NF-κB in 7.19 cells.

Distribution of *gslA* among various strains of *P. gingivalis*. Interestingly, the *gslA* gene is not found in the genome of *P. gingivalis* W83 (16). A DNA region (7.7 kb) in the ATCC 33277 genome that contains *gslA* between direct repeats (TTTTA TAA) was deleted in the W83 genome (Fig. 6A). To investigate the presence of *gslA* in other *P. gingivalis* strains, Southern blot analysis was performed. Three probes for *gslA* hybridized to the genomic DNA from *P. gingivalis* strains ATCC 33277, TDC117, TDC275, and SU63 but not to genomic DNA from strains W83, TDC60, and GAI-7802 (Fig. 6B).

DISCUSSION

We purified cell surface components of KDP136 and found two proteins with molecular masses of 123 and 43 kDa in the fraction with high ability to induce NF-κB activation in 7.19

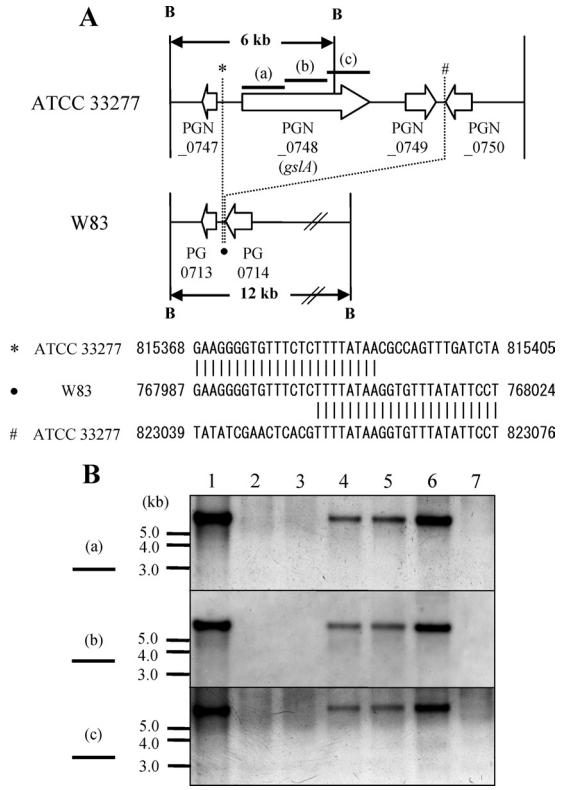

FIG. 6. Distribution of *gslA* among various strains of *P. gingivalis*. Hybridization probes a, b, and c for internal regions of *gslA*, described in Materials and Methods, are indicated in both panels. (A) A DNA region (7.7 kb) in the ATCC 33277 genome that contains *gslA* between direct repeats (TTTTATAA) was deleted in the W83 genome. B, restriction enzyme-recognized site for BamHI; ⋆, #, and ●, detailed DNA sequences of indicated regions are shown below. Arrows show CDSs. PGN numbers are CDS numbers from *P. gingivalis* ATCC 33277. PG numbers are those of *P. gingivalis* W83. (B) Chromosomal DNA from ATCC 33277 and other strains of *P. gingivalis* was digested with BamHI and subjected to Southern hybridization using the probes mentioned above. Lanes: 1, *P. gingivalis* ATCC 33277; 2, W83; 3, TDC60; 4, TDC117; 5, TDC275; 6, SU63; 7, GAI-7802.

cells. In Western blot analysis, mouse antiserum that could inhibit the activation of NF-κB in 7.19 cells reacted to the 123-kDa protein but not to the 43-kDa protein. When 7.19 cells were stimulated with freeze-dried *P. gingivalis*, a *gslA* deletion mutant lacking the 123-kDa protein did not induce NF-κB activation in 7.19 cells, whereas a *pgm6* or *pgm6 pgm7* deletion mutant lacking the 43-kDa protein induced NF-κB activation. These results all indicate that the 123-kDa protein encoded by *gslA* is a crucial factor for inducing NF-κB activation in 7.19 cells.

The polyclonal antibody against the 123-kDa protein partially inhibited the activation of NF-κB in 7.19 cells induced by freeze-dried KDP136, indicating that the 123-kDa protein was involved in the induction of NF-κB activation in 7.19 cells. The partial inhibition might be due to the existence of another gingipain-sensitive ligand that could induce NF-κB activation in 7.19 cells. In accordance with this hypothesis, stimulation with the *gslA* deletion mutant induced a slightly higher level of NF-κB activation in 7.19 cells than that induced by stimulation with the wild-type strain.

The lysate of the gingipain-null mutant KDP136 contained a much larger amount of the 123-kDa protein than did the lysate of the wild-type strain ATCC 33277. In addition, the Triton X-114-extracted cell surface protein fraction of ATCC 33277 had no 123-kDa protein, whereas that of KDP136 did have the 123-kDa protein. It is likely that the 123-kDa protein was degraded by surface-associated gingipains after transportation of the protein to the cell surface in the wild-type *P. gingivalis*. The degradation of the 123-kDa protein on the cell surface of wild-type *P. gingivalis* is consistent with the findings by other investigators that gingipains cleave and process the products encoded by the *fimA*, *mfa1*, *rgpA*, *rgpB*, *kgp*, *hagA*, and *ragA* genes, which are located on the bacterial cell surface (5, 12, 24). Masuda et al. reported that the expression of mRNA for gingipains is modulated by environmental stress (9). The expression and activities of gingipains gradually decreased with the increase of the growth rate of *P. gingivalis*. The activities of gingipains in the culture fluids of *P. gingivalis* under aerated conditions were approximately eightfold lower than those in anaerobic conditions. In those conditions, the 123-kDa protein may remain intact on the cell surface and induce NF-κB activation even in wild-type *P. gingivalis* cells.

The physiological function of the 123-kDa protein encoded by *gslA* is unknown, and no homologue to GslA was found by BLASTP analysis. Interestingly, *gslA* was found in four of seven strains of *P. gingivalis*. Although strain W83 has been found to be more virulent than strain ATCC 33277 in a mouse subcutaneous chamber model, rats challenged with strain ATCC 33277 had more periodontal bone loss than those challenged with other strains (4, 6). Although further studies are needed to determine whether the presence of *gslA* is influential in the pathogenesis of *P. gingivalis*, the restricted distribution of *gslA* might be associated with the virulence potential of each strain. The common features of the four strains possessing *gslA* could be a clue for finding the pathogenic function of GslA.

The 123-kDa protein could induce NF-κB activation in 7.19 cells in a TLR2- and TLR4-independent manner. Although the activity to induce NF-κB activation was analyzed using only 7.19 cells, it is important to determine whether and how this ligand stimulates host cells derived from human periodontal tissue. Supposing that GslA is able to induce NF-κB activation in periodontal tissue, it would lead to the secretion of proinflammatory cytokines and chemokines that promote the acceleration of inflammatory responses in periodontal tissue. Regulation of gingipain expression by environmental conditions may affect this process.

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