**Fusobacterium nucleatum** Envelope Protein FomA Is Immunogenic and Binds to the Salivary Statherin-Derived Peptide

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We have previously shown that one of the minimal active regions of statherin, a human salivary protein, for binding to *Fusobacterium nucleatum* is a YQPVPE amino acid sequence. In this study, we identified the FomA protein of *F. nucleatum*, which is responsible for binding to the statherin-derived YQPVPE peptide. Overlay analysis showed that a 40-kDa protein of the *F. nucleatum* cell envelope (40-kDa CE) specifically bound to the YQPVPE peptide. The equilibrium association constant between the affinity-purified 40-kDa CE and the YQPVPE peptide was 4.30 × 10⁴. Further, the purity and amino acid sequence analyses of the purified 40-kDa CE revealed approximately 98.7% (wt/wt) purity and a high degree of homology with FomA, a major porin protein of *F. nucleatum*. Thus, a FomA-deficient mutant failed to bind to the YQPVPE peptide. In addition, increased levels of a FomA-specific mucosal IgA antibody (Ab) and plasma IgG and IgA Abs were seen only in mice immunized nasally with cholera toxin (CT) and the purified 40-kDa FomA protein. Interestingly, saliva from mice that received FomA plus CT as a mucosal adjuvant nasally prevented *in vitro* binding of *F. nucleatum* to statherin-coated polystyrene chloride plates. Taken together, these results suggest that induction of specific immunity to the 40-kDa FomA protein of *F. nucleatum*, which specifically binds to the statherin-derived peptide, may be an effective tool for preventing the formation of *F. nucleatum* biofilms in the oral cavity.

Bacterial adherence is often an essential first step in the colonization and establishment of an infection in a susceptible host. Thus, the adherence of bacteria is itself an important virulence factor. *Fusobacterium nucleatum* is a Gram-negative anaerobe that plays a pivotal role in early colonization during dental plaque formation. Thus, it is known that *F. nucleatum* bridges not only salivary proteins and other coaggregating oral bacterial strains but also early and late colonizers in the oral cavity (4, 22). In addition, it has been shown that the presence of *F. nucleatum* is a predisposing factor for some systemic diseases, such as urinary tract infections (32) and intrauterine infections associated with preterm birth (23), as well as for oral diseases, including alveolar abscesses (35) and periodontal disease (18). One may assume that the oral cavity, which is covered with a mucosal membrane, is the most likely portal of entry into the host for this pathogenic organism.

Among human salivary proteins, statherin is known as a unique acidic, carbohydrate-free phosphoprotein (14) that inhibits the primary and secondary precipitation of calcium salts. In addition, statherin is tightly adsorbed to enamel surfaces (13) and facilitates adhesion by *F. nucleatum* (41), *Actinomyces viscosus* (28), *Actinomyces naeslundii* (28), and Porphyromonas gingivalis (1, 2) to its preadsorbed hydroxyapatite surface. Our previous study also showed that of all human salivary proteins, statherin exhibited the strongest ability to bind to *F. nucleatum* cell surface protein (33).

In order to elicit maximal levels of antigen (Ag)- or pathogen-specific immune responses in both mucosal and systemic lymphoid tissue compartments, it is necessary to use an appropriate mucosal adjuvant (9). Nasal immunization has emerged as perhaps the most effective regimen for inducing both peripheral and mucosal immunity, including salivary secretory IgA (S-IgA) antibody (Ab) responses (25). It is now well accepted that cholera toxin (CT), produced by *Vibrio cholerae*, is the most potent mucosal adjuvant for the induction of Ag-specific Ab responses when coadministered with a protein Ag (42). Thus, detailed studies have shown that CT induces CD4⁺ T helper type 2 (Th2) cells for the induction of mucosal S-IgA, systemic subclass IgG1 and IgG2b, and systemic IgE Ab responses (38, 42, 44). Among Th2 cytokines, interleukin-4 (IL-4) notably plays a key role in the mucosal adjuvanticity of CT (29, 39). Further, our recent study showed that nasal application of CT as an adjuvant enhanced mucosal S-IgA Ab responses to a T-cell-independent Ag through cross talk between IL-5 receptor-positive (IL-5R⁺) B-1a B cells and IL-5-producing CD4⁺ T cells (15).

We have previously shown that two amino acid alignments (YQPVPE and PYQPQYQ) on the statherin molecule are the most likely segments that bind to *F. nucleatum* (33). In this study, we show that the 40-kDa FomA protein of *F. nucleatum* is responsible for interaction with the YQPVPE peptide in the active binding segment of the statherin molecule. In addition, we examined whether induction of FomA-specific Ab
responses could prevent the binding of F. nucleatum to the YQPVPE peptide. The outcomes of these studies could lead to the development of effective strategies for the prevention of F. nucleatum adherence and infection as well as for the prevention of its associated diseases.

MATERIALS AND METHODS

Bacterial culture conditions and radiolabeling. F. nucleatum ATCC 25586 (wild type) was grown in Trypticase soy broth (Becton Dickinson, Sunnyvale, CA) supplemented with yeast extract (1 mg/ml), hemin (5 μg/ml), and menadione (1 μg/ml) at 37°C under anaerobic conditions (80% N₂, 10% CO₂, 10% H₂) (33). Before the bacteria were harvested, the cells were washed three times with 50 mM phosphate-buffered saline (PBS; pH 7.2) and were suspended in the same buffer. In some experiments, the harvested cells were radiolabeled with the Bolton-Hunter reagent kit (PerkinElmer Japan Co., Ltd., Yokohama, Japan). The specific activity of the iodinated protein was 1.7 mCi (58.1 MBq) per 10⁹ cells.

FomA-deficient mutant of F. nucleatum. The internal sequence of the fomA gene, which encodes the major porin protein of F. nucleatum, was amplified by PCR. Two primers (primer fomAEcoR2) were used for PCR were 5'-CGG CAT CTC CAG CCT GGA GAC CAA ATG G-3' (primer fomABamFI1) and 5'-GGG ATC CCC AAC AAC TCC ACT ATT ATG TCC-3' (primer fomAEcoR2). These primers were inserted at BamHI or EcoRI restriction sites, respectively. The PCR product was digested with BamHI and EcoRI and was then ligated into the suicide vector pSF151 (30). The resultant plasmid, pFOMA151, was electrotransformed into F. nucleatum strain SN-3. Cells were electroporated by a method described previously, with minor modifications (8). Briefly, the harvested F. nucleatum cells were washed with 10% glycerol, and the resultant plasmid, pFOMA151 (5 μg/100 μl of cell suspension), was pulsed with a Bio-Rad Gene Pulser II (1.8 kV, 25 μF, 400 Ω, and 7 ms) into the competent cells, which were then incubated on ice for 5 min. The cell suspension was then added to 4.0 ml of TSB medium and was incubated at 37°C overnight. The fomA mutant strain SN-3 was subsequently isolated on kanamycin-containing agar plates.

Mice. Female C57BL/6 mice (6 to 8 weeks old) were purchased from Japan SLC Inc. (Hamamatsu, Japan). These mice were maintained in the experimental animal facility at Osaka University (Saita, Japan), and all experiments were conducted in accordance with the guidelines provided by the Osaka University Graduate School of Dentistry Animal Care Committee.

Preparation of bacterial envelopes and biotinylation of the synthesized peptide. F. nucleatum whole cell envelopes (CEs) were prepared by a method described previously, with minor modifications (36). Briefly, the harvested cell suspension was ultracentrifuged in an ice bath at 1 min intervals with an ultrasonic disperser (UR-200P; Tomy Seiko, Tokyo, Japan) emitting 200 W and was allowed to cool for 1 min between ultrasonic treatments. After undissociated cells were removed by centrifugation (1,000 × g), the supernatants were centrifuged at 100,000 × g for 90 min in order to recover the whole CEs. The supernatant fraction containing polysaccharide components and the whole CEs was washed three times with 50 mM PBS (pH 7.2) followed by distilled water and was then lyophilized. Analogous peptides (YQPVPE) corresponding to the amino acid sequence of statherin were commercially synthesized and purified at the Sequence and Peptide Synthesis Facility of Aves Labs, Inc. (Tigard, OR). The amino acid sequences and mass values of products were confirmed by mass spectrometry as well as analytical high-pressure liquid chromatography. The synthesized YQPVPE peptide was biotinylated using the ECL protein biotinylation module according to the manufacturer’s instructions (GE Healthcare UK Ltd., Buckinghamshire, United Kingdom).

Western blot ligand overlay assay. In order to examine whether F. nucleatum whole CEs possessed the ability to bind the YQPVPE peptide, a ligand overlay assay was performed using the biotinylated YQPVPE peptide (33). F. nucleatum whole CEs were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a 15% polyacrylamide gel (Bio-Rad Laboratories, Hercules, CA) and were then transferred to a nitrocellulose membrane. The membrane was then incubated with 1 mg/ml of PBS solution of the biotinylated YQPVPE peptide at 4°C overnight, followed by incubation with horseradish peroxidase (HRP)-conjugated streptavidin (GE Healthcare UK Ltd.). The F. nucleatum CEs that were bound to the YQPVPE peptide were detected by using an HRP-conjugated substrate kit (Bio-Rad Laboratories, Hercules, CA).

Purification of the F. nucleatum CE protein that binds to the YQPVPE peptide. The YQPVPE peptide-conjugated affinity column was prepared according to the manufacturer’s instructions for CNBr-activated Sepharose 4B (GE Healthcare UK Ltd.) in order to purify CEs binding to the YQPVPE peptide. In brief, yophilized whole CEs dissolved in 10 mM Tris-HCl buffer (pH 6.7) were applied to the YQPVPE affinity column. The column was washed with 10 volumes of Tris-HCl (pH 6.7), and the components bound to the affinity column were eluted with Tris-HCl containing 0.5 M NaCl. The affinity column purification was repeated in order to obtain highly purified CE. Purified CEs were subjected to the ligand overlay assay in order to confirm their ability to bind to the YQPVPE peptide. The purity (wt/wt) of the YQPVPE-binding CE was calculated by the Experion system (Bio-Rad Laboratories) based on SDS-PAGE.

Biomolecular interaction analysis. The affinity of binding between the YQPVPE peptide-binding F. nucleatum CE and the synthesized YQPVPE peptide was analyzed by a BIACore model X system (GE Healthcare UK Ltd.) as described previously (37). One hundred micrometers per milliliter of purified YQPVPE peptide-binding CE or bovine serum albumin (BSA) (Sigma, St. Louis, MO) diluted in 10 mM sodium acetate (pH 4.0) was applied to the surface of a CMS sensor chip (GE Healthcare UK Ltd.). The synthesized YQPVPE peptide, as an analyte, was diluted with HBSP buffer (10 mM HEPES, 150 mM NaCl, 3.4 mM EDTA, and 0.005% Tween 20 [pH 7.4]) and was then injected at a flow rate of 30 μl/min at 25°C. The concentration of the analyte was adjusted to 1.3, 2.5, 5, 10, and 20 mM. Binding between the purified CE or BSA and the YQPVPE peptide was monitored and presented as resonance units (RU) in a sensorgram. Analysis of these kinetic parameters was conducted with the BIAevaluation software package, version 3.1 (GE Healthcare UK Ltd.), according to the operator’s manual.

Amino acid sequence analysis of a 40-kDa CE protein purified by YQPVPE peptide-conjugated column chromatography. A 40-kDa CE protein purified by YQPVPE peptide-conjugated column chromatography was subjected to amino acid sequence analysis (Nippi Research Institute, Biomatrix, Tokyo, Japan). The amino acid sequences of the fragments digested with the BSA were determined by the Edman degradation method (6) and were deposited in the BLASTp database.

Dot blot assays for binding to the YQPVPE peptide. To examine the direct binding of wild-type F. nucleatum and the ΔfomA mutant strain SN-3 to the YQPVPE peptide, a dot blot assay was performed as described previously (33). Briefly, cells of both wild-type F. nucleatum and the ΔfomA mutant strain SN-3 were washed with KCl buffer (50 mM KCl solution containing 1 mM KH₂PO₄, 1 mM CaCl₂, and 0.1 mM MgCl₂ [pH 6.8]) and were immobilized on an Immobilon-P polyvinylidene difluoride (PVDF) membrane with a Bio-Dot apparatus (both from Bio-Rad Laboratories). The membrane was blocked with 5% Block Ace and was incubated overnight at room temperature with 3 ml of 125I-labeled wild-type F. nucleatum or the ΔfomA-labeled ΔfomA mutant strain SN-3. Subsequently, the membrane was washed with KCl buffer containing 100 mM NaCl and was subjected to autoradiography. The binding activity toward the YQPVPE peptide or BSA was determined by the relative densitometric analysis of reaction dots by a PowerPC Mac with the NIH Image program, version 1.62 (National Institutes of Health, Bethesda, MD).

Nasal immunization with the F. nucleatum FomA protein. In order to harvest the lipopolysaccharide (LPS)-free purified FomA protein as an immunogen, yophilized whole CEs were applied three times to the YQPVPE peptide-conjugated column purification column, and the LPS was further removed using a polyoxymethylenyldiurea (PVDf) membrane with a Bio-Dot apparatus (both from Bio-Rad Laboratories). The membrane was blocked with 5% Block Ace and was incubated overnight at room temperature with 3 ml of 125I-labeled wild-type F. nucleatum or the ΔfomA-labeled ΔfomA mutant strain SN-3. Subsequently, the membrane was washed with KCl buffer containing 100 mM NaCl and was subjected to autoradiography. The binding activity toward the YQPVPE peptide or BSA was determined by the relative densitometric analysis of reaction dots by a PowerPC Mac with the NIH Image program, version 1.62 (National Institutes of Health, Bethesda, MD).

Sample collection. Plasma and stimulated saliva were collected on days 0 and 21. Stimulated-saliva samples were collected following intraepithelial injection of mice with pilocarpine (Sigma) as described previously (16, 42). Mice were sacrificed 7 days after the last immunization (day 21), and nasal wash specimens were obtained by instillation of 1 ml of PBS three times into the posterior opening of the nasopharynx with a 30-gauge hypodermic needle (11).

Mucosal and systemic 40-kDa FomA-specific Ab assays. Levels of FomA-specific Abs in plasma and external secretions were determined by an enzyme-linked immunosorbent assay (ELISA) (11, 43). Briefly, 96-well Falcon microtiter plates (BD Biosciences, San Jose, CA) were coated with 1 μg/ml FomA protein in PBS. After wells were blocked with 1% BSA in PBS, 2-fold serial dilutions of samples were added and incubated overnight at 4°C. HRP-labeled goat anti-mouse μ, γ, or a heavy-chain-specific Abs (Southern Biotechnology Associates, Birmingham, AL) were added to individual wells, followed by
three washes with PBS-Tween 20 (0.05%). For IgG Ab subclass analysis, biotinylated monoclonal Abs (M Abs) specific for IgG1, IgG2a, IgG2b, or IgG3 (BD Biosciences PharMingen, Franklin Lakes, NJ) and a peroxidase-conjugated goat antibod Ab (Vector Laboratories, Burlingame, CA) were used for detection. The color reaction was developed for 15 min at room temperature with 100 μl of 1.1 mM 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid). End point titer were expressed as the reciprocal log 2 of the last dilution that gave an optical density at 415 nm 0.1 greater than the background. To enumerate the Ab-forming cells (AFCs), mononuclear cells from the spleen and cervical lymph nodes (CLNs) were isolated aseptically by a mechanical dissociation method using gentle teasing through stainless steel screens as described previously (16, 43). A modified dissociation method based on a previously described protocol (11, 16) was used to isolate nasal passages (NPs). Mononuclear cells from submandibular glands (SMGs) and nasopharynx-associated lymphoreticular tissues (NALTs) were isolated by an enzymatic dissociation procedure with collagenase type IV (0.5 mg/ml; Sigma), followed by discontinuous Percoll gradient centrifugation (GE Healthcare UK Ltd.) (11, 16). Mononuclear cells obtained from mucosal and systemic lymphoid tissues were subjected to an enzyme-linked immunospot (ELISPOT) assay in order to determine the numbers of Ag-specific AFCs (11, 16).

F. nucleatum biofilm formation assay on statherin-coated PVC plates. The assay of inhibition of static F. nucleatum biofilm formation on statherin was performed by using 96-well polyvinyl chloride (PVC) plates according to the method of O’Toole and Kolter (31). Briefly, stationary-phase cultures of F. nucleatum were washed three times and resuspended at a concentration of 5 × 10^9 cells/ml with KCl buffer (80 mM KCl solution containing 1 mM KH₂PO₄, 1 mM CaCl₂, and 0.1 mM MgCl₂, pH 6.7). The YQPVPE peptide (25 μg; 100 μl) or stimulated-saliva samples (diluted 1:10 with KCl buffer; 100 μl) from either naive mice or mice given the 40-kDa FomA protein nasally, with or without CT, were then incubated with F. nucleatum (5 × 10^10 cells) at 25°C for 3 h. Subsequently, each mixture was added to statherin-coated PVC plates (100 μg/ml KCl buffer) and was incubated at 25°C overnight. The plates were then washed with KCl buffer, and the wells were stained with 25 μl of 1% crystal violet (CV) and incubated for 15 min. The stained biofilm was extracted in 95% ethanol and diluted (2-fold), and biofilm formation was assessed using the Biacore X biomolecular interaction monitor of interactions between the purified 40-kDa CE and the F. nucleatum outer membrane.

Statistical analysis. The results are expressed as the mean ± standard error of the mean (SEM) or 1 standard deviation (SD). All mouse groups were compared with control mice by using an unpaired Mann-Whitney U test with Statview software (Abacus Concepts, Berkeley, CA) designed for Macintosh computers. P values of <0.05 or <0.01 were considered significant.

RESULTS

Isolation and identification of F. nucleatum CE protein binding to the YQPVPE peptide, the minimal active segment. In order to identify F. nucleatum components that specifically bind to the YQPVPE peptide, whole CEs of F. nucleatum were subjected to a Western blot assay. The YQPVPE peptide specifically bound to a 40-kDa protein of the F. nucleatum CE (40-kDa CE) (Fig. 1A). Further, we purified the 40-kDa CE from the F. nucleatum whole CE fraction by repeated elution of whole CEs through stainless steel screens as described previously (16, 43). A modified dissociation procedure with collagenase type IV (0.5 mg/ml; Sigma), followed by discontinuous Percoll gradient centrifugation (GE Healthcare UK Ltd.) (11, 16). Mononuclear cells obtained from mucosal and systemic lymphoid tissues were subjected to an enzyme-linked immunospot (ELISPOT) assay in order to determine the numbers of Ag-specific AFCs (11, 16).

Amino acid sequence of the 40-kDa CE protein. We next determined the amino acid sequence of the 40-kDa CE by Edman degradation (6). When the 40-kDa CE was specifically digested with V8 protease, the amino-terminal sequences of the three digested fragments were determined to be VAPAWRPNGS, KF XFRLYQXK, and LNXXYNNYNVX, respectively. A homology search for the amino acid sequence in the BLASTp database revealed that the 40-kDa CE had a high degree of homology (94%) with FomA, known as a porin protein of F. nucleatum. A modified dissociation procedure with collagenase type IV (0.5 mg/ml; Sigma), followed by discontinuous Percoll gradient centrifugation (GE Healthcare UK Ltd.) (11, 16). Mononuclear cells obtained from mucosal and systemic lymphoid tissues were subjected to an enzyme-linked immunospot (ELISPOT) assay in order to determine the numbers of Ag-specific AFCs (11, 16).

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Statistical analysis. The results are expressed as the mean ± standard error of the mean (SEM) or 1 standard deviation (SD). All mouse groups were compared with control mice by using an unpaired Mann-Whitney U test with Statview software (Abacus Concepts, Berkeley, CA) designed for Macintosh computers. P values of <0.05 or <0.01 were considered significant.

RESULTS

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Real-time profiles of the affinity of binding of the YQPVPE peptide to the 40-kDa CE protein. To determine the affinities of interactions between the purified 40-kDa CE and the YQPVPE peptide, direct surface plasmon resonance was assessed using the Biacore X biomolecular interaction monitoring system. The YQPVPE peptide, as an analyte, bound to the immobilized 40-kDa CE with a high rate of association and a low rate of dissociation compared with binding to the control (BSA) (Fig. 2). The values of the association rate constants (kₐss [1/M·s]), dissociation rate constants (kₘₐ₈ [1/s]), and equilibrium association constants (Kₐ) were determined as kₐss/kₘₐ₈. The values of the association rate constants (kₐss [1/M·s]), dissociation rate constants (kₘₐ₈ [1/s]), and equilibrium association constants (Kₐ) were calculated as kₐss/kₘₐ₈.

FIG. 1. The YQPVPE peptide binds to the 40-kDa cell envelope (CE) protein of F. nucleatum. (A) The F. nucleatum (ATCC 25586) CEs that specifically bind to the YQPVPE peptide were determined by a ligand overlay assay. F. nucleatum whole CEs were separated by SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was incubated with the biotinylated YQPVPE peptide followed by horseradish peroxidase (HRP)-conjugated streptavidin for substrate development. Lane 1, molecular mass standard marker; lane 2, F. nucleatum whole CEs. (B and C) SDS-PAGE (B) and ligand overlay analysis (C) of F. nucleatum CEs purified by a YQPVPE peptide-coupled, CNBr-activated Sepharose 4B column. Lanes 1 and 3, molecular mass standard marker; lane 2, YQPVPE affinity column-purified F. nucleatum; lane 4, purified F. nucleatum CE incubated with the biotinylated YQPVPE peptide.

Amino acid sequence of the 40-kDa CE protein. We next determined the amino acid sequence of the 40-kDa CE by Edman degradation (6). When the 40-kDa CE was specifically digested with V8 protease, the amino-terminal sequences of the three digested fragments were determined to be VAPAWRPNGS, KF XFRLYQXK, and LNXXYNNYNVX, respectively. A homology search for the amino acid sequence in the BLASTp database revealed that the 40-kDa CE had a high degree of homology (94%) with FomA, known as a porin protein of F. nucleatum (Fig. 3). Taken together, these observations demonstrate that the YQPVPE peptide binds specifically to FomA, a major protein of the F. nucleatum outer membrane.

Targeted mutagenesis of fomA. In order to confirm that FomA specifically binds to statherin, we next constructed a FomA-deficient mutant of F. nucleatum. Targeted inactivation of the fomA gene in F. nucleatum strain ATCC 28856 resulted in the generation of the ∆fomA mutant strain SN-3 (Fig. 4A). The mutation was confirmed by PCR analysis of chromosomal DNA with primers fomABamF1 and apHA3F2 (5′-TCC GTA TCT TTT ACG CAG CGG-3′). A fragment spanning the region between fomA and apHA3 was amplified using these primer pairs (Fig. 4B); further examination showed that only a single insert was present in the chromosome, and no transcript was made from the mutant gene (data not shown). Thus, the target gene, fomA, was inactivated in this mutant. We compared the growth rate of the ∆fomA mutant strain SN-3 with that of ATCC 25586 and confirmed that the presence of an antibiotic cassette in the chromosome did not influence the growth rate (data not shown).
Wild-type _F. nucleatum_ but not the ΔfomA mutant strain SN-3 binds to the YQPVPE peptide on PVDF membranes. To examine whether the ΔfomA mutant strain SN-3 bound the YQPVPE peptide, a PVDF membrane adsorbed with the YQPVPE peptide or BSA was incubated with either 125I-labeled wild-type _F. nucleatum_ or the 125I-labeled ΔfomA mutant strain SN-3. The binding activity was visualized by autoradiography, and the relative density of reaction dots was determined by densitometric analysis. The ΔfomA mutant strain SN-3 exhibited reduced levels of binding to the YQPVPE peptide (21%) (Fig. 4C) when the rate of dot density representing wild-type _F. nucleatum_ binding to the YQPVPE peptide had been adjusted to 100%. These results suggest that FomA plays a key role in the binding of _F. nucleatum_ to the YQPVPE amino acid sequences of the statherin molecule.

**Induction of FomA-specific immunity.** We next examined whether simultaneous nasal administration of LPS-free, purified FomA plus CT as a mucosal adjuvant would enhance FomA-specific immune responses in both mucosal and systemic lymphoid tissues. One microgram of the purified FomA protein contained 0.1 endotoxin unit (EU). Mice given FomA protein plus CT nasally exhibited significantly higher Ag-specific S-IgA Ab responses in saliva and SMGs than mice given FomA alone nasally (Fig. 5A and B). In addition, FomA-specific S-IgA Ab responses were noted in nasal secretions (Fig. 5A). The laminae propriae of NPs (Fig. 5B) and NALT (Fig. 5C) of mice given FomA plus CT nasally also exhibited elevated AFC responses. Elevated FomA-specific plasma IgA and IgG Ab responses were also seen in mice given nasal FomA plus CT compared with those of mice nasally immunized with FomA alone (Fig. 6A). Thus, significantly increased numbers of FomA-specific IgA and IgG AFCs were seen in the spleens of mice nasally immunized with FomA plus CT (Fig. 6B). IgG subclass Ab analyses revealed that levels of both FomA-specific IgG1 and IgG2b Abs were markedly higher than those for control mouse groups (Fig. 6A). In contrast, relatively low IgG2a Ab responses and no IgG3 Ab responses were seen (Fig. 6A). Taken together, these results show that

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**FIG. 2.** Biomolecular interactions of affinity column-purified _F. nucleatum_ CEs and the YQPVPE peptide. The affinity of binding of the YQPVPE peptide to immobilized _F. nucleatum_ CEs (A) or BSA (B) was estimated by the BIACore system. HBSP buffer (10 mM HEPES, 150 mM NaCl, 3.4 mM EDTA, and 0.005% Tween 20 [pH 7.4]) was used as a running buffer, with a flow rate of 30 µl/min. The statherin peptide solution at various concentrations (1.3–20 µM) was monitored and injected over the 40-kDa CE (100 µg/ml) or BSA (100 µg/ml) on the CM5 sensor chip.

**FIG. 3.** Homology search for the amino acid alignment of the 40-kDa _F. nucleatum_ component. The 40-kDa CE of _F. nucleatum_ was digested with V8 protease, and the amino-terminal sequences of the three cleaved fragments were determined using the BLASTp database program. The underlined sequences correspond to the identified amino-terminal sequences of _F. nucleatum_ FomA and the 40-kDa CE of the _F. nucleatum_ envelope obtained from the YQPVPE peptide-conjugated affinity column.
nasal administration of FomA plus CT as a mucosal adjuvant effectively induced FomA-specific Ab responses in the oral mucosae as well as in other mucosal and systemic immune compartments.

Inhibition of biofilm formation by saliva from mice given FomA plus CT nasally. Since F. nucleatum is known to be one of the microorganisms observed microscopically at the axis of dental plaque, and since it readily assembles into the dental plaque biofilm, the inhibitory effects of saliva from mice nasally immunized with FomA plus CT on biofilm formation were examined. Saliva from naïve mice or from mice immunized with FomA alone inhibited biofilm formation minimally (17% and 34%, respectively) (Fig. 7). In contrast, saliva from mice immunized with FomA plus CT showed significant inhibition.

FIG. 4. Construction and functional analysis of the ΔfomA mutant strain SN-3. (A) pFOMA151 contains an internal fragment of fomA and a kanamycin resistance gene (aphA3). SN-3 was produced by single-crossover recombination. (B) The ΔfomA mutant strain SN-3 and the wild-type strain of F. nucleatum (ATCC 25586) were subjected to PCR with forward primer fomABamF1 and reverse primer aphA3F2. (C) Dot blot assay for direct binding activity of ATCC 25586 or mutant strain SN-3 to the YQPVPE peptide. The dot blots show typical results for both the experimental (binding to the YQPVPE peptide) and control (binding to BSA) groups. The level of dot blot density for the control group was subtracted from the level of dot blot density for the experimental group in each experiment. The relative percentage of dot density for the control group was calculated relative to the dot density for wild-type F. nucleatum bound to the YQPVPE peptide, which was defined as 100%. The graph shows the average percentages of dot density for the binding of wild-type F. nucleatum (100%) or SN-3 (21%) to the YQPVPE peptide. The experiments were performed in triplicate on three separate occasions. Data are expressed as means ± standard deviations. The asterisk indicates a significant difference (P < 0.05) from the result for the wild-type strain.

FIG. 5. FomA-specific immune responses in external secretions and mucosal lymphoid tissues. C57BL/6 mice were nasally immunized weekly for three consecutive weeks either with FomA protein (20 μg) plus cholera toxin (CT; 1 μg) as a mucosal adjuvant (filled bars) or with FomA only (open bars). (A) Seven days after the last immunization, the levels of FomA-specific IgA Abs in nasal washes and saliva were determined by FomA-specific ELISAs. Data are means ± SEMs (n = 15). Double asterisks indicate significant differences (P < 0.01) from results for control mice. (B and C) Seven days after the last immunization, mononuclear cells isolated from NPs, SMGs, and NALT were subjected to Ag-specific ELISPOT assays in order to determine the numbers of IgG and IgA AFCs. Mice immunized nasally with FomA alone were used as controls. Data are means ± SEMs (n = 15). Asterisks indicate significant differences (**, P < 0.01) from results for control mice.
of biofilm formation (82%) (Fig. 7). The synthetic YQPVPE peptide solution (final concentration, 125 μg/ml) showed moderate inhibition of biofilm formation on statherin-coated PVC plates (53%) (Fig. 7). Taken together, these results showed that F. nucleatum biofilm formation is prevented by saliva containing significant levels of FomA-specific S-IgA Abs.

DISCUSSION

We have previously suggested that F. nucleatum and statherin bind through a protein-protein interaction. In this regard, YQPVPE (amino acids [aa] 21 to 26) and PYQPQYQ (aa 33 to 39) were the minimal active regions of the salivary statherin molecule necessary for binding to F. nucleatum (33). This study is the first to show that a 40-kDa protein of the F. nucleatum cell envelope (40-kDa CE) is the major component for binding to salivary statherin. This protein molecule showed high homology with FomA, known as a porin protein in the F. nucleatum outer membrane. In addition, significant levels of Ag-specific S-IgA Ab responses were induced in both mucosal and systemic lymphoid tissues when mice were nasally immunized with FomA plus native CT as a mucosal adjuvant. Interestingly, saliva from mice given FomA plus CT significantly inhibited the formation of F. nucleatum biofilms on statherin-coated PVC plates. Since the attachment of F. nucleatum to the surfaces of teeth and oral mucosae plays a central role in plaque formation and the subsequent development of F. nucleatum-associated periodontitis, as well as playing roles in some systemic diseases, the present study has shed light on the mechanisms of F. nucleatum colonization and the possible prevention of the diseases it mediates.

The present study initially focused on the elucidation of the specific binding site of F. nucleatum to the YQPVPE peptide on statherin, and sequencing analysis showed that the 40-kDa CE specifically bound to the YQPVPE peptide (Fig. 1). When the affinity of binding between the 40-kDa CE and the YQPVPE peptide was examined by surface plasmon resonance technology using a BIAcore system, the values of the associa-
intrauterine infections associated with preterm birth (23), as well as in systemic diseases, such as urinary tract infections (32) and saliva is of central importance in the prevention of

Streptococcus sanguinis (17) and P. gingivalis (19, 20). To further confirm the specific binding of FomA to the YQPVPE peptide, we constructed a ΔfomA mutant strain of F. nucleatum, SN-3. When we examined the interactions between F. nucleatum and the statherin-derived YQPVPE peptide, the ΔfomA mutant strain SN-3 clearly showed a level of binding to the statherin peptide significantly lower than that of the wild-type strain (Fig. 4C), although SN-3 maintained a low affinity for the YQPVPE peptide, confirming previous reports that F. nucleatum possesses a variety of lectin-like and non-lectin-like adhesins that are used to attach to host cells (12), extracellular matrix proteins (40), and salivary proteins (33). The YQPVPE peptide-binding activity of SN-3 may be due to these other molecules. Nevertheless, our results clearly showed that the ΔfomA mutant strain SN-3 displayed a significant reduction in binding to the YQPVPE peptide, suggesting that F. nucleatum FomA is most likely an important bacterial anchor or receptor for adherence to teeth and to the oral mucosal surface and thus that it plays a role in subsequent biofilm formation.

It is well known that mucosal immunization results in protective immunity in both mucosal and systemic compartments (24). Among all potential immunization routes, the nasal Ag delivery system is presumably the most efficacious regimen for inducing Ag-specific S-IgA Ab responses in the oral cavity (26). In this regard, nasal administration of purified FomA protein plus native CT induced Ag-specific IgA Ab responses in saliva and SMGs (Fig. 5A and B). Importantly, saliva that contained FomA-specific S-IgA Abs effectively inhibited the binding of F. nucleatum to statherin for the biofilm formation (Fig. 7). However, nasal vaccination with FomA alone did not induce mucosal or systemic immune responses (Fig. 5 and 6). Thus, saliva from mice nasally immunized with FomA alone failed to inhibit biofilm formation (Fig. 7). These results indicate that FomA itself, without CT as a mucosal adjuvant, is not a strong immunogen. Indeed, in support of this view, F. nucleatum is one of many commensal organisms in the oral cavity. Still, since F. nucleatum acts as a bridge between salivary proteins and other coaggregating strains of oral bacteria for plaque formation with early colonizers (i.e., Streptococcus gordoni and Streptococcus oralis) and late colonizers (i.e., P. gingivalis and Actinomyces naeslundii) (4, 22), the induction of FomA-specific S-IgA Abs in saliva is of central importance in the prevention of F. nucleatum colonization. In addition, F. nucleatum is believed to be a pathogen in systemic diseases, such as urinary tract infections (32) and intrauterine infections associated with preterm birth (23), as well as in several oral diseases (20, 35). Thus, induction of FomA-specific S-IgA Abs in saliva appears to be important for the prevention of biofilm formation and of F. nucleatum-associated oral and systemic disease development. To support this view, it has been shown that Ag-specific S-IgA Abs provide effective protective immunity against bacterial (34) and viral (7) pathogens.

Since whole saliva contains Abs from gingival crevicular fluid that originate from serum IgG Abs, it is possible that salivary FomA-specific IgG Abs, as well as S-IgA Abs, are important. Indeed, nasal immunization with FomA plus CT induced significantly increased FomA-specific IgG Ab responses in plasma (Fig. 6A). Further, plasma from mice nasally immunized with FomA plus CT partially inhibited the binding of F. nucleatum to statherin (data not shown). Despite this potential, we did not detect FomA-specific IgG Abs in saliva. Therefore, one could conclude that FomA-specific S-IgA Abs are the major players for the prevention of F. nucleatum-associated biofilm formation in the oral cavity.

Our results clearly showed that the use of CT as a mucosal adjuvant is required for the induction of FomA-specific Ab responses in both mucosal and systemic compartments. However, nasal application of enterotoxins, such as CT and Escherichia coli heat-labile enterotoxin (LT), has been shown to be inappropriate for humans due to central nervous system (CNS) toxicity, resulting in the induction of Bell’s palsy (5). In this regard, our group has constructed mutants of CT harboring single-amino-acid substitutions in the ADP-ribosyltransferase active center that render them nontoxic (43) and has developed a novel nontoxic chimeric mucosal adjuvant that combines the nontoxic subunit A of mutant CT (E112K) with the pentameric subunit B of LT from enterotoxigenic E. coli (mCT-A/LT-B) (27, 43). In addition, it has been reported that other nontoxic nasal adjuvants, including a plasmid expressing H3 ligand cDNA (pFL) and the CpG oligodeoxynucleotide (ODN), successfully induced Ag-specific S-IgA Ab responses in saliva (10, 16). In this regard, we are currently assessing the efficacy of a combined nasal vaccine, i.e., FomA protein with nontoxic mutant CT (mCT-A/LT-B), pFL, and the CpG ODN, for the prevention of oral infection with F. nucleatum.

In summary, the present study identified the 40-kDa CE of F. nucleatum specifically binding to the YQPVPE peptide as FomA, an F. nucleatum outer membrane protein. In this regard, saliva containing FomA-specific S-IgA Abs from mice nasally immunized with FomA protein plus CT markedly inhibited the formation of F. nucleatum biofilms on statherin-coated PVC plates. Understanding of the cellular and molecular mechanisms of initial F. nucleatum colonization in the oral cavity could lead to the development of immunobiological strategies to prevent not only F. nucleatum infection but also its associated oral and systemic diseases.

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