Purification and Characterization of Fibroblast-Activating Factor Isolated from *Porphyromonas gingivalis* W50

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A 24-kDa polypeptide which activated the incorporation of [3H]thymidine into human fibroblasts was isolated from the outer membrane vesicles of *Porphyromonas gingivalis* W50. This polypeptide, named fibroblast activating factor (FAF), was isolated by 3-(3-cholamidopropyl)-dimethylammonio]-1-propane-sulfonate (CHAPS) detergent extraction and purified by DEAE ion-exchange chromatography and preparative isoelectric focusing. Purified FAF (100 ng of protein per ml) caused a 400% increase in [3H]thymidine incorporation into human gingival fibroblasts (HGFs) compared with results for controls. FAF was characterized as (i) a polypeptide with molecular masses of 24 kDa when heated at 100°C for 5 min and 44 kDa when unheated, (ii) heat sensitive but not affected by selected reducing reagents, and (iii) possessing slight phosphatase activity. N'-terminal sequence analysis revealed no homology with *P. gingivalis* peptides or with any host-derived growth factors. These data suggest that FAF functions as a significant virulence factor which in vivo is capable of modulating homeostasis in local connective tissues.

Periodontal disease represents a group of chronic inflammatory diseases which for the most part have a bacterial etiology (41, 43). The developing periodontal pocket is populated by a specific and predictable microbiota, with selected members initiating inflammatory events characteristic of this disease. An examination of the literature concerned with the in vivo host-parasite interactions which occur between the pathogenic periodontal microbiota and the host (both human and animal) reveals little definitive information of the actual in vivo events which occur in a host relative to the progression of periodontal disease. Thus, while we have a large amount of data on the in vitro virulence characteristics of this microbiota, we still have very little information relevant to the in vivo events which occur in the susceptible host. One member of this putative periodontopathic microbiota, *Porphyromonas gingivalis*, has been the subject of numerous investigations, including human clinical studies (26, 37, 42, 52), animal models of periodontal disease progression (16), and physiological-biochemical studies of its virulence potential (27). In human and animal studies, *P. gingivalis* remains undetected in the healthy periodontium but rises to significant levels in the diseased state (44). In vitro, *P. gingivalis* synthesizes a diverse number of potential in vivo virulence factors, including fimbriae (7, 9, 53), lipopolysaccharides (LPS) (13), hemagglutinins (27, 34), and several proteases (2, 11, 12, 28-31, 38, 45, 50, 51). If active in a susceptible host, these virulence factors could result in a rapid and significant destruction of the structural integrity of the periodontium as well as a significant systemic infection. Since this does not occur, it is therefore important to determine which virulence determinants are produced in vivo and whether they are able to alter the physiological conditions of diseased sites by attacking the cells in connective tissues directly or by indirectly modifying local immune responses.

Fibroblasts play a significant role in the maintenance of the integrity of a variety of tissues, including the periodontium. One of the functions of fibroblasts is the continued regeneration of periodontal tissue. Therefore, any factor(s) which affects the quality or functional capabilities of fibroblasts may affect the integrity of the tissue and structure of the periodontium. A large number of the early studies of the interaction of periodontopathic bacteria with fibroblasts involved studies of the effects of whole-cell extracts on specific fibroblast functions (18, 19, 40, 46). These initial studies progressed to studies of the role of LPS (1, 20, 22, 23, 32), proteases (51), and fimbriae (14) as modulators of fibroblast function. The *P. gingivalis* LPS-fibroblast interaction studies revealed that LPS inhibits normal mouse and rat fibroblast function by inducing gamma interferon (8), interleukin-1 (IL-1), and IL-6 (48). A 35-kDa *P. gingivalis*-derived protein (trypsinlike protease) induced collagenase production and plasminogen activator in human gingival fibroblast (HGF) cultures (51), while the *P. gingivalis* fimbriae were capable of inducing thymocyte activation factor in HGFs (14).

No studies have concentrated on the role of *P. gingivalis* cell surface molecules (excluding LPS) in modulating HGF growth. The results presented here describe the isolation, purification, and characterization of an outer membrane-associated protein which functions to significantly enhance HGF proliferation. We refer to this protein as fibroblast-activating factor (FAF).

MATERIALS AND METHODS

Bacterial strains and culturing conditions. *P. gingivalis* W50 (ATCC 53978), W83, and ATCC 33277 and *Bacteroides fragilis* ATCC 25285 were used. All strains were stored either frozen at −70°C or lyophilized. For use, the cultures were thawed or rehydrated in reduced transport fluid (24) and were grown initially on the surface of enriched Trypticase soy agar (ETSA) plates (47). Culture purity was determined by Gram staining and by conformity to recognized biochemical reactions. For liquid growth, cells were removed from the ETSA surface and transferred to 2% (wt/vol) mycosoma broth medium (BBL, Becton Dickinson, Cockeysville, Md.) supplemented with 5 μg of hemin and 1

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μg of menadione per ml. All cultures were grown at approximately 37°C in a Coy anaerobic chamber in an atmosphere of 80% N2 plus 10% CO2 plus 10% H2.

Preparation of MF. P. gingivalis W50 grown as described above was used for the isolation of both the membrane fraction (MF) and outer membrane vesicles (OMV) (Fig. 1). For the isolation of these surface components, P. gingivalis was routinely grown in large batch culture (i.e., 16 to 20 liters). Whole cells were collected by centrifugation (23,000 x g for 15 min), and the cell pellets were resuspended in phosphate-buffered saline (PBS; pH 7.4) and washed once with PBS. The washed cells were sedimented at 23,000 x g for 15 min and resuspended in PBS containing (final concentrations) 2 mM phenylmethylsulfonyl fluoride (PMSF), 2 mM benzamidine, and 2 mM Na-p-tosyl-L-lysine chloromethyl ketone (TLCK). The cells were thoroughly mixed and immediately broken in a cold Aminco French pressure cell at 17,000 lb/in2 (SLM Instruments, Urbana, Ill.). The cell suspension was subjected to at least eight rounds of disruption until greater than 99% of the cells were disrupted (as revealed by phase-contrast microscopy). Whole cells and cell debris were removed by centrifugation (23,000 x g for 20 min), and the MF was sedimented from the supernatant at 165,000 x g for 1.5 h. The MF was washed twice by ultracentrifugation in PBS (pH 7.4) and sterilized by filtration through a 0.2-μm-pore-size filter unit (Gelman Sciences, Ann Arbor, Mich.). The sterilized MF was stored at −20°C until used.

Preparation of OMV. P. gingivalis W50 cells were separated from the culture supernatant by Pellicon ultrafiltration through a 0.45-μm-pore-size filter cassette (Millipore, Bedford, Mass.) (Fig. 1). An OMV-enriched fraction was collected by ultrafiltration of the supernatant through a Pellicon cassette with a molecular weight cutoff of 300,000 (Millipore). The OMV were sedimented from the OMV-enriched fraction by ultracentrifugation (165,000 x g for 1.5 h), and the vesicles were washed at least three times to remove soluble medium components. The resulting OMV fraction was suspended in PBS and stored at −20°C. The yield of OMV was approximately 5 mg of protein per liter of P. gingivalis.

Purification of FAF from OMV. In initial studies, FAF was purified from both the isolated MF and the OMV. Since chemical and morphological analyses revealed the OMV to be homogeneous in appearance and chemically devoid of contaminating peptidoglycan components and nucleic acid, the FAF was isolated exclusively from the OMV fraction (Fig. 1).

(i) Solubilization of FAF from OMV. The zwitterionic detergent 3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate (CHAPS; Pierce, Rockford, Ill.) was used to extract the FAF from the OMV. CHAPS was capable both of solubilizing the FAF and of having minimal cytotoxic activity for HGFs (data not shown). Approximately 80 mg of OMV protein was suspended in 20 ml of 50 mM Tris-HCl buffer (pH 7.4), and the suspension was mixed thoroughly and drawn into a syringe. The OMV were sequentially extruded through 18- to 27-gauge hypodermic needles. CHAPS was added to the homogenous OMV suspension to a final concentration of 5% (wt/vol), and the suspension was sonicated at full-power output with a cell disrupter (model 200; Branson Sonication, Danbury, Conn.) for 10 min. The sample was cooled continuously in an ice bath during sonication. After sonication, the sample was incubated at 37°C for 4 h with gentle shaking. Insoluble material was removed by ultracentrifugation (165,000 x g for 2 h), and the CHAPS-insoluble fraction was stored at −20°C. The CHAPS-soluble fraction was either used immediately for FAF purification or stored at −20°C until used.

(ii) DEAE ion-exchange column chromatography. The CHAPS-soluble fraction was adjusted with 50 mM Tris-HCl (pH 7.4) buffer to produce a CHAPS concentration of 1% (wt/vol). Approximately 50 ml of sample was applied to a DEAE-Sephacel column (2.5 by 16 cm; Pharmacia Fine Chemical, Uppsala, Sweden) equilibrated with 50 mM Tris-HCl (pH 7.4) containing 1% (wt/vol) CHAPS. After being washed with 200 ml of the Tris-CHAPS buffer at an elution rate of 50 ml/h, protein fractions were eluted from the column by a 250-ml NaCl gradient (0 to 0.2 M). Protein elution was monitored with an ISCO model UA-5 UV monitor at an optical density of 280 nm. All procedures were carried out at 4°C.

Assays of protein content and FAF activity (see below) and analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were performed on alternating fractions which had been dialyzed against PBS (pH 7.4) for at least 16 h. FAF was eluted through a 0.2-μm-pore-size filter (Gelman Sciences).

(iii) Preparative IEF. Fractions which showed maximum protein and FAF activity from the DEAE ion-exchange chromatography were further purified by a Rotofor preparative electrofocusing column (Bio-Rad Laboratories, Richmond, Calif.). Samples for Rotofor analysis (see Fig. 2 [peak]) were dialyzed against distilled water at 4°C and brought to a final volume of 50 ml containing 1% (wt/vol) CHAPS, 0.5% (wt/vol) amphoteries (pH 3.5 to 10; Bio-Lyte; Bio-Rad), 0.5% (wt/vol) amphoteries (pH 4 to 6), and 10% (vol/vol) glycerol in distilled water. The electrolytes in the anode and cathode chambers were 0.1 M H2PO4 and 0.1 M NaOH, respectively. Isoelectric focusing (IEF) in the Rotofor cell was accomplished at 12 W of constant power at an initial voltage of 600 V, at 4°C for 5 h. The focusing was continued until the voltage had stabilized (1,250 V) for at least 30 min. The isoelectrofocused fractions were collected, pH values were measured, and volumes were determined. The amphoteries and glycerol were removed by dialysis against PBS (pH 7.4). FAF activity, protein content, and SDS-PAGE analysis were carried out for each of the fractions to determine both the distribution of protein and the amount of FAF activity.
HGFs. Normal HGFs isolated and purified by standard procedures were used (49). Cells were cultivated in Dulbecco modified Eagle's medium (DMEM) supplemented with 2 mM L-glutamine (Sigma Chemicals, St. Louis, Mo.), 10% fetal bovine serum (FBS; Hazelton, Lenaxa, Kans.), 100 IU of penicillin (JRH Biosciences, Lenaxa, Kans.) per ml, 100 µg of streptomycin (JRH Biosciences) per ml, and 0.25 µg of amphotericin B (JRH Biosciences) per ml at 37°C in CO₂-humidified air. HGFs between passages 4 and 12 were used.

Measurement of FAF activity. FAF activity was measured as the amount of [³H]thymidine incorporated into both subconfluent and confluent HGFs. Fibroblasts were seeded at a concentration of 10⁵ cells per well in 96-well microculture plates (Corning) in DMEM supplemented with 10% (vol/vol) FBS and were incubated in a CO₂-humidified 37°C incubator for 24 h. The DMEM was removed by aspiration and replaced with fresh DMEM containing the test fractions. The FBS concentration was reduced to 0 during the experimental period (24 h). During the final 6 h of the incubation, 0.5 µCi of [³H]thymidine (specific activity, 74 GBq/mmol; ICN Radiochemicals, Irvine, Calif.) was added to each well. At the end of the 24-h incubation period, the HGFs were detached with 0.25% trypsin and 0.02% EDTA (JRH Biosciences) and harvested on a glass fiber filter (Cambridge Technology, Watertown, Mass.) with a cell harvester (Otto Hiller, Madison, Wis.), and the distribution of radioactivity was measured with a liquid scintillation counter (model LS1; Beckman, Palo Alto, Calif.).

All HGF assays were run in triplicate, and the results were expressed as mean stimulation indices (SI) ± standard errors as follows: SI = [³H]thymidine uptake (counts per minute)/[³H]thymidine uptake (counts per minute) in control medium. The statistical significance of the difference between test and control group results was examined by Student's t test.

Fibroblast proliferation. HGF proliferation was measured by counting the total number of fibroblasts. HGFs (5 × 10⁴ cells per 500 µl per well) were seeded in 24-well culture plates in DMEM plus 10% FBS. The cells were cultured overnight to allow attachment, and the medium was exchanged for fresh DMEM (FBS free) containing FAF. Cells were incubated for an additional 24, 48, and 72 h and then removed from the plate with a trypsin-EDTA mixture (see above). Aliquots were placed into plastic tubes and stained with trypan blue (GIBCO, Grand Island, N.Y.), and HGF viability and cell number were determined with a Neubauer hemocytometer. Experiments were carried out in quadruplicate, and significance was calculated by Student's t test.

One- and two-dimensional SDS-PAGE analysis. The relative molecular migration, purity, and biophysical characteristics of FAF were examined by one- and two-dimensional SDS-PAGE analysis in 12% acrylamide (17). All gels were stained either with Coomassie brilliant blue R250 or with silver (4). The relative molecular weight of FAF was determined by comparison with Bio-Rad SDS-PAGE molecular weight standards. For the two-dimensional analyses, we used the basic procedure of Hindahl and Iglewski (15). Purified native FAF (unheated, without 2-mercaptoethanol [2-ME]) was applied to an SDS-PAGE gel and electrophoresed (see above). Each lane of the native gel was cut into a 1-cm width and heated at 100°C for 10 min in sample buffer with 2-ME. The denatured gel strips were juxtaposed horizontally along the top of a 1-cm stacking gel, and the denatured proteins were separated by the standard procedure described above. Protein purity was determined by spot size, distribution, and conformation. Heat modifiability was determined by measuring relative migration off the protein diagonal. Relative molecular weight was determined by comparison with Bio-Rad low- and high-molecular-weight standards run in the same gel. Effect of selected treatments on purified FAF. Twenty-five micrograms of FAF protein per ml was resuspended in sterile PBS (pH 7.4) and incubated at the temperatures and/or with the chemicals indicated in Table 2 for 1 h. The reactions were stopped by cooling the mixtures to 4°C, after which dialysis and measurement of [³H]thymidine incorporation by HGFs were performed. To prevent a direct effect on the HGF assay itself, this high FAF concentration (25 µg of protein per ml) was used, and FAF activity was subsequently measured at 125 ng of protein per ml. We observed no cytotoxic effects against HGF by any of the reagents used.

Neutralizing assay with immunoglobulin G anti-IL-1α or -β. To examine whether endogenous IL-1 affected FAF activity, FAF was incubated with purified goat anti-human recombinant IL-1α/β immunoglobulin G (R&D Systems, Minneapolis, Minn.) for 1 h and neutralization was determined by the FAF assay.

Determination of enzyme activity in purified FAF. The distribution of selected enzymatic activities in the purified FAF was measured with the API ZYM system (API Laboratory Products, Plainview, N.Y. [21]). The results were expressed as follows (nanomoles of substrate reacted): −−, 0; +, 5; ++, 10; ++++, 20; +++++, 30; +++++++, >40. The enzymes and their substrates are listed in Table 3.

Determination of protein content. The quantitative protein content of all fractions was measured with the bicinchoninic acid protein assay kit (Pierce). Relative protein content was also measured by spectrophotometric absorption at 280 nm in a Beckman model DU-65 spectrophotometer.

Amino acid sequencing. N-terminal sequencing of the pure proteins was performed after transfer to Problott membrane (Applied Biosystems, Foster City, Calif.) as described by

### Table 1. Purification of FAF from P. gingivalis W50

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total vol (ml)</th>
<th>Total amt of protein (mg)</th>
<th>Total FAF activity (U)*</th>
<th>Sp act (U/mg)</th>
<th>Purification (fold)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OMV</td>
<td>20</td>
<td>80</td>
<td>32,000</td>
<td>400</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>CHAPS-solubilized fraction</td>
<td>26</td>
<td>6.5</td>
<td>20,809</td>
<td>3,200</td>
<td>8</td>
<td>65</td>
</tr>
<tr>
<td>DEAE-Sepahell</td>
<td>20</td>
<td>0.76</td>
<td>16,000</td>
<td>21,052</td>
<td>52.6</td>
<td>50</td>
</tr>
<tr>
<td>Preparative IEF</td>
<td>2.6</td>
<td>0.06</td>
<td>2,080</td>
<td>33,333</td>
<td>83.3</td>
<td>6.5</td>
</tr>
</tbody>
</table>

*Values are from bicinchoninic acid assay.

Each value is the reciprocal of the highest dilution producing one-half of the maximum enhancement effect times the total volume.
Matsudaira (25). Pure FAF (approximately 30 μg), treated with sample buffer at 60°C for 10 min was loaded onto a 12% acrylamide gel. After electrophoresis, the protein solution was transferred onto Problott membrane according to the manufacturer’s instructions. The membrane was stained with Coomasie brilliant blue R250 and destained with 50% ethanol and 10% acetic acid. The target FAF protein band was excised from the dried membrane, and its amino acid sequences were determined by the Biomolecular Resources Center at the University of California at San Francisco. Samples were subjected to Edman degradation with an Applied Biosystems 470A gas-phase sequencer.

Other materials. P. gingivalis W50 LPS was extracted by cold MgCl₂-ethanol precipitation (4). P. gingivalis 381 purified fimbriae were provided by F. Yoshimura. Phenol-water-extracted LPS preparations of Escherichia coli 0127:B8 were purchased from Sigma Chemicals.

RESULTS

Purification of the P. gingivalis W50 FAF. Both P. gingivalis MF and P. gingivalis OMV fraction possessed FAF activity (data not shown). To avoid outer membrane-associated peptidoglycan and cytoplasmic contamination during the purification procedures, OMV were used for all FAF isolations. Solubilization of 80 mg of OMV protein with 5% CHAPS resulted in an 8-fold increase in FAF specific activity, with an almost 53- and 83-fold purification after DEAE-Sephacel and preparative IEF, respectively (Table 1). DEAE-Sephacel ion-exchange chromatography of the CHAPS-soluble OMV fraction resulted in the elution of the FAF activity in a major protein peak at a NaCl concentration of approximately 0.05 M (Fig. 2). Preparative IEF of the peak protein fractions from the DEAE-ion-exchange chromatography (Fig. 2 [peak]) resulted in the recovery of approximately 70% of the FAF activity in one fraction with a pI point of approximately 5.0 (Fig. 3).

SDS-PAGE analysis of the distribution of polypeptides during FAF purification steps (Table 1) is seen in Fig. 4. Initially, the OMV contained at least nine major polypeptides with relative molecular masses of 48, 42, 35, 29, 27, 26, 23, 19, and 18 kDa (Fig. 4, lane 1). CHAPS solubilization resulted in the removal of at least four of these major OMV polypeptides (29, 27, 26, and 23 kDa) (Fig. 4, lane 2). Note that a 24-kDa band is apparent. SDS-PAGE of the DEAE-ion-exchange column chromatography fractions resulted in the selective elution of three polypeptides (48, 35, and 24 kDa) in the peak fraction (Fig. 4, lane 5). These three major polypeptides were eluted selectively (Fig. 4, lanes 3 to 7) by 0.05 M NaCl. SDS-PAGE of IEF fractions 9 and 10 (Fig. 4, lanes 11 and 12) resulted in the separation of a single polypeptide with a relative molecular mass of approximately 24 kDa (Fig. 4).

FIG. 2. DEAE-Sephacel ion-exchange chromatographic separation of FAF from CHAPS-soluble OMV fraction. Approximately 6.5 mg of CHAPS-soluble protein was applied to the DEAE-Sephacel column (2.5 by 16 cm; equilibrated with 50 mM Tris-HCl [pH 7.4] plus 1% CHAPS), and FAF was eluted with a NaCl gradient at 4°C. Peak reveals fractions associated with high FAF activity. The protein concentration (optical density at 280 nm [OD280]) in each fraction was determined. FAF specific activity (FAF-SPEC) was determined from FAF activity of the serially diluted fractions by the following formula: FAF-SPEC = FAF-U/OD280, where FAF-U = (maximum reciprocal dilution in which SI > 3) × SI.

FIG. 3. Rotofor IEF of DEAE-Sephacel chromatographic fractions. Peak fractions from DEAE-Sephacel ion-exchange chromatography were dialyzed against distilled water. The dialyzate was resuspended in distilled water plus 1% CHAPS, 0.5% ampholyte (pH 3.5 to 10), 0.5% ampholyte (pH 4 to 6), and 10% glycerol (total volume, 50 ml). The protein was focused at 12 W of constant power (as described in the text) for 5 h at 4°C. See the legend to Fig. 2 for definition of FAF-SPEC.

FIG. 4. SDS-PAGE profile of the distribution of P. gingivalis W50 OMV protein during FAF purification. Lanes: 1, crude OMV (100 μg); 2, CHAPS-solubilized OMV (20 μg); 3 to 7, DEAE-ion-exchange chromatographed peak fractions (50 μl for each fraction); 8 to 13, fractions 6 to 11 from IEF (50 μl for each fraction). All samples were heated at 100°C for 10 min and loaded in a 12% acrylamide gel silver nitrate stain.
4, lane 11) when heated for 10 min at 100°C. This 24-kDa polypeptide corresponded to all of the FAF activity seen in Fig. 3. Figure 5A shows the effect of 2-ME on the FAF protein under unheated conditions. Both bands (in the presence and absence of 2-ME) revealed relative molecular masses of 44 kDa. Two-dimensional SDS-PAGE analysis (Fig. 5B) revealed one staining polypeptide at the cross-point with molecular masses of 24 and 44 kDa in the heated and in the unheated samples, respectively. Silver staining of both the one- and two-dimensional SDS-PAGE gels revealed a single polypeptide, with no LPS or outer membrane protein contamination observed (data not shown).

N'-terminal amino acid analysis. The N'-terminal amino acid sequence of the 24-kDa polypeptide is shown in Fig. 6. Preliminary homology data indicate a 47% homology with the Staphylococcus aureus fibronectin-binding protein sequence.

FAF activity and HGFs. (i) FAF activity of P. gingivalis strains and culture supernatants. Whole cells of P. gingivalis W50, W83, and ATCC 33277 as well as their spent growth supernatants (OMV-containing fraction) had significant proliferative effect on all HGFs (Fig. 7). The FAF activity in the spent growth supernatant was all associated with the OMV fraction after ultracentrifugation of the supernatant. Maximum FAF activity occurred in the P. gingivalis strains at a 1:100 dilution of the culture supernatant, and activity was reduced at a 1:1,000 dilution. At a low dilution (1:10), there was an inhibitory effect by the mycoplasma medium itself (see the legend to Fig. 7). This tendency was also detected in whole-cell samples. B. fragilis ATCC 25285 was devoid of FAF activity in both the whole cells and the supernatant.

(ii) Effect of FAF on proliferation of HGFs. The effect of purified FAF in 0, 1, and 10% FBS on HGF proliferation was determined over a 72-h period by direct cell count (Fig. 8). The FBS concentration had a significant effect on HGF proliferation, with increasing concentrations resulting in an increase in the number of cells. In the presence of 10% FBS, approximately three times more HGFs occurred than in the control (0% FBS) at 72 h. However, addition of FAF (300 ng of protein per ml) to HGFs grown at 0 and 1% FBS concentrations resulted in a significant proliferative effect compared with that of the control.

(iii) Comparison of FAF activity with fimbrae and LPS. Purified fimbrae from P. gingivalis 381 W50 and Escherichia coli O127:B8 (Sigma) LPS had essentially no fibroblast-activating ability compared with controls at all concentrations tested compared with FAF itself (Fig. 9). FAF did display a marked activity in a dose-dependent fashion between 0.08 and 0.6 μg of FAF protein per ml (Fig. 9).

(iv) Effect of selected treatment on FAF. The effects of selected chemical and physical treatments on the purified FAF are indicated in Table 2. FAF lost its activity by heat treatment even at 60°C. Treatment with various protease inhibitors or activators resulted in either negligible or no change in FAF activity. Soybean trypsin inhibitor and

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FIG. 7. Distribution of FAF activity ([3H]thymidine incorporation into HGFs) in whole cells and spent growth supernatant of P. gingivalis strains and B. fragilis ATCC 25285. Supernatants were sterilized with 0.2-μm-pore-size filters and applied to HGF cultures at 1:10, 1:100, and 1:1,000 dilutions of the original. Totals of 10^8, 10^9, and 10^10 cells of each bacterial strain were applied to HGF cultures. Results are expressed as SI (counts per minute of experimental results/counts per minute of DMEM control results). FAF activity mean values were as follows (± standard errors [SE]): DMEM control (no addition), 2,705.6 ± 61.7 cpm; 1:10 diluted mycoplasma medium control, 559.0 ± 70.7 cpm (0.21 in SI); 1:100 diluted mycoplasma medium control, 55.61 ± 4.55 cpm (0.94 in SI); 1:1,000 diluted mycoplasma medium control, 3,013.5 ± 184.1 cpm (1.11 in SI). Values that were significantly different from control values are indicated as follows: at level *, P < 0.05; **, P < 0.01.

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FIG. 6. N'-terminal amino acid sequence of 24-kDa protein purified from P. gingivalis W50.
cysteine resulted in 37 and 23% inhibitions of FAF activity, respectively. Anti-IL-1 immunoglobulin Ga/β was not capable of neutralizing FAF activity, suggesting that IL-1 is not related to growth enhancement by FAF.

**Enzymatic activity in purified FAF.** The distribution of enzyme activity in the purified FAF is indicated in Table 3. Only negligible phosphatase and phosphohydrolase activities were apparent. No protease activities in the purified FAF were detected (assayed only after FAF isolation in the absence of protease inhibitors).

**DISCUSSION**

A variety of host- and bacterially derived factors which activate fibroblasts have been discussed in regard to their relationship to periodontal disease (49). The study reported here describes the first outer membrane protein from *P. gingivalis* which has been shown to have a significant fibroblast-proliferative effect. Because of its activity on fibroblasts, we refer to it as a FAF. Previous reports have described the growth or proliferation of fibroblasts as a function of a variety of complex and mixed factors. These include dental plaque (9, 10), cell extracts (20, 40, 46), and LPS (1, 8, 22, 23). The crude spent growth supernatant also showed an HGF effect, depending on the protein concentrations (Fig. 7). At high supernatant protein concentration, [1H]thymidine incorporation into the HGFs was clearly inhibited. There was not only an inhibition of cell proliferation, but also a change in cell morphology from the normal elongated shape to round (51). More than likely, this cytotoxic effect at high concentrations of spent growth superna-

**TABLE 2. Effect of selected treatments on FAF from* P. gingivalis* W50**

<table>
<thead>
<tr>
<th>Treatment of FAF</th>
<th>Conc</th>
<th>FAF activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heat (15 min at 60°C)</td>
<td>10 μg/ml</td>
<td>-</td>
</tr>
<tr>
<td>Heat (15 min at 80°C)</td>
<td>10 μg/ml</td>
<td>-</td>
</tr>
<tr>
<td>Heat (15 min at 100°C)</td>
<td>10 μg/ml</td>
<td>-</td>
</tr>
<tr>
<td>Dithiothreitol</td>
<td>10 mM</td>
<td>89.9</td>
</tr>
<tr>
<td>EDTA</td>
<td>10 mM</td>
<td>100.0</td>
</tr>
<tr>
<td>EGTA</td>
<td>10 mM</td>
<td>99.5</td>
</tr>
<tr>
<td>Soybean trypsin inhibitor</td>
<td>20 μg/ml</td>
<td>63.5</td>
</tr>
<tr>
<td>Leupeptin</td>
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</tr>
<tr>
<td>Benzamidine</td>
<td>2 mM</td>
<td>81.2</td>
</tr>
<tr>
<td>AEBSF</td>
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</tr>
<tr>
<td>Cysteine</td>
<td>10 mM</td>
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<tr>
<td>Anti-IL-1α immunoglobulin G</td>
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<tr>
<td>Anti-IL-1β immunoglobulin G</td>
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* Percent activity retained compared with control (no FAF); final concentration of FAF, 1.25 μg/ml (1:200 dilution of each sample).

**TABLE 3. Enzyme activity detected in purified FAF of* P. gingivalis* W50**

<table>
<thead>
<tr>
<th>Enzyme assay</th>
<th>Substrate</th>
<th>Activity in FAF</th>
</tr>
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<tbody>
<tr>
<td>Leucine aminopeptidase</td>
<td>L-Leucyl-naphthylamide</td>
<td>-</td>
</tr>
<tr>
<td>Valine aminopeptidase</td>
<td>L-Valyl-naphthylamide</td>
<td>-</td>
</tr>
<tr>
<td>Cysteine aminopeptidase</td>
<td>L-Cystyl-2-naphthylamide</td>
<td>-</td>
</tr>
<tr>
<td>Trypsin</td>
<td>N-Benzoyl-DL-arginine-naphthylamine</td>
<td>-</td>
</tr>
<tr>
<td>Chymotrypsin</td>
<td>N-Glutaryl-phenylalanine-2-naphthylamine</td>
<td>-</td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td>2-Naphthyl-phosphate</td>
<td>±</td>
</tr>
<tr>
<td>Acid phosphatase</td>
<td>2-Naphthyl-phosphate</td>
<td>+</td>
</tr>
<tr>
<td>Phosphohydrolase</td>
<td>Naphthyl-AS-BS-phosphate</td>
<td>+</td>
</tr>
</tbody>
</table>

* Enzyme activity was measured with the API ZYM system (see Materials and Methods). Purified FAF (2 μg of protein) was used for each enzyme assay.

FIG. 8. Effect of FAF on proliferation of HGFs in the presence and absence of FBS. HGFs (5 × 10⁴/500 μl) were cultured overnight and then stimulated with nothing (○) or with FAF (30 ng/ml) (■), FAF (300 ng/ml) plus 10% FBS (△), or 1% FBS (□) in DMEM. Cultivation was continued up to 72 h. Viable numbers of HGFs were measured at 0, 24, 48, and 72 h after stimulation. Values were significantly different from those for nonstimulated HGF.

FIG. 9. Comparison of *P. gingivalis* (Pg) W50 purified FAF, *P. gingivalis* 381 fimbriae, *P. gingivalis* W50 LPS, and *E. coli* 0127:B8. All samples were applied to HGF cultures at the indicated concentrations, and the effects on the incorporation of [1H]thymidine into HGFs were determined. Results were expressed as SI (± standard error [SE]). Control value (no addition), 1,666.8 ± 70.8 cpm. **, significant (P < 0.01) differences.
tant was due to mycoplasma broth proteins in the culture medium. The supernatant effect was also inhibited by the addition of protease inhibitors (data not shown), suggesting some proteolytic enzyme activity in the spent growth supernatant. Whole cells which were washed extensively with PBS displayed significant FAF-enhancing activity even at low doses (see Fig. 7). Larjava et al. (20) have also suggested this dual effect (inhibition-enhancement) in their data. However, our observation with FBS-depleted HGFs was different from those of the other reports, which for the most part employed high FBS concentrations in their proliferative assays. In the presence of FBS, there appeared to be some masking of the FAF activity (data not shown); however, even in the presence of 10% FBS, there was still significant FAF-enhanced [3H]thymidine incorporation compared with that in the control. Depletion of FBS in the culture medium resulted in a rate of incorporation of [3H]thymidine into HGFs at FAF concentrations of 150 ng/ml (Fig. 9) that was four to five times higher than that of the control (minus FAF). Purified fimbriae and LPS had no FAF ability compared with the purified FAF (Fig. 9). In fact, at high doses (100 μg/ml) of P. gingivalis and E. coli LPS, [3H]thymidine uptake by the HGFs was inhibited (see also Barthold and Millar [1]).

Recently, Uitto et al. (51) showed that a protease derived from P. gingivalis had an enhancing effect on the production of collagenase and plasminogen activator by HGFs. Bacterial proteases (i.e., trypsin, papain, and ficin) have been demonstrated to be capable of modulating the growth of various host cells (39) including fibroblasts (5, 33). Interestingly, several of these molecules were found to possess insulinomimic properties (3, 35, 36). Blumberg and Robbins (3) found that the activation of fibroblasts by proteases was due to a modification of a cell surface protein, more than likely fibronectin. Uitto et al. (51) also suggest that a P. gingivalis-derived protease (35 kDa) modified an HGF-associated fibronectin which resulted in a fractional alteration in the HGFs. Thrombin, well-known as a host factor (for wound healing), also has serine protease activity (6). To eliminate this activity by FAF, we examined the effect of several protease inhibitors on FAF activity and enzyme activity (Tables 2 and 3, respectively). There was a very low to negligible level of phosphatase activity; however, no trypsin, chymotrypsin, or other proteolytic activities were ever found in any of the FAF preparations examined. This fact suggests that (i) FAF does not include trypsin activity, (ii) the trypsinlike enzyme found in P. gingivalis is not associated with FAF, and (iii) of the protease inhibitors, only soybean trypsin inhibitor was slightly inhibitory for purified FAF. The inhibitory effect of the soybean trypsin inhibitor is more than likely due to some serine protease contamination, since several other serine protease inhibitors (AEBSF [Calbiochem] and leupepsin) did not show any inhibitory activity. The relationship between phosphatase activity and host cell membrane modification is still unclear. Interestingly, homology searches of the N-terminal amino acid sequence data revealed that the FAF molecule possessed 47% homology with a fibronectin-binding protein precursor. Therefore, FAF might function to modify the HGF fibronectin in a manner similar to that of serine proteases.

Since FAF was found to be active on both the membrane and OMV, it is possible that FAF plays a significant role in the modulation of HGF activity, especially in proliferation. The small size (20- to 500-nm diameter) of the OMV provides them with easy access to host tissues therefore and the ability of FAF and other OMV-associated molecules to act either directly or indirectly in the host tissues. However, future studies will be required to determine the exact location of FAF in the host and its in vivo function.

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