Signal Peptide Analyses and Localization of 40-kDa Outer Membrane Protein in *Porphyromonas gingivalis* 381

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Abstract

A 40-kDa outer membrane protein (HBP35) has been molecularly cloned from the periodontal pathogen *Porphyromonas gingivalis* 381. The predicted amino acid sequence of HBP35 consists of 344 amino acid residues, having a molecular weight of 37,559. In the latest computational analysis, a typical signal peptide was suggested to be present at the amino terminus of the protein. On Western blot analysis using an antibody against recombinant HBP35 (rHBP35), two protein bands were seen in crude extracts of the recombinant *Escherichia coli*. In addition, a lower molecular weight band was seen in the periplasmic fraction. The partial amino acid sequence of the lower molecular weight HBP35 was N-QELKTSADMKGsfKKNVLE-C at the amino terminus and corresponded to the 22nd to 41st amino acids of the HBP35 protein. This indicates that there is a cleavage site for the signal peptide between the 21st and the 22nd residues. Immunostaining with the anti-rHBP35 antibody showed that the HBP35 protein was mainly located outside of the *P. gingivalis* cytoplasm. Taken together, the results suggest that HBP35 passes through the inner membrane after cleavage of the signal peptides at the N-terminal of the protein and is bound to the outer membrane of this bacterium.

Keywords :

Porphyromonas gingivalis, signal peptides, outer membrane, localization

Introduction

Porphyromonas gingivalis is a major pathogen in severe forms of periodontal disease, and is also related to non-oral diseases such as cardiovascular disease and osteoporosis (1). This Gram-negative anaerobic rod possesses several virulence factors, including fimbriae, proteinases, hemagglutinin, lipopolysaccharide and outer membrane vesicles (1-7).

A 40-kDa outer membrane protein has been molecularly cloned from periodontal pathogen *P. gingivalis* 381 as an *EcoRI* DNA fragment (8). It is specifically expressed in *P. gingivalis* cells (9, 10). Hemin is able to bind to the recombinant 40-kDa outer membrane protein in a dose-dependent manner, indicating that it is a heme-binding protein (11). Thus, the outer membrane gene and protein are designated *hbp35* and HBP35, respectively. The

HBP35 protein is associated with various pathogenicity pathways in *P. gingivalis*, and we previously demonstrated that HBP35 plays a role in adherence to host cells, coaggregation with other bacteria, and hemagglutination (10, 12-16).

A DNA sequence analysis of the 2004 bp *EcoRI* fragment revealed that the open reading frame of the *hbp35* gene is from 499 to 1540. A promoter is present in the sequence upstream of the translation start codon (ATG), and -35 and -10 regions are TTGCAG (position 411 to 416) and TATTTT (position 434 to 439), respectively. The transcription start site (+1) is also predicted to be located 53 nt upstream of the translation start codon. The palindromic sequence is from 1554 to 1601 in the 3'-non-coding region, and a typical rho-independent transcription terminator is present in the palindromic sequence region (17). The predicted HBP35 protein

consists of 344 amino acid residues with a calculated molecular weight of 37,559, and computer analysis suggests the presence of a signal peptide (11).

Many bacterial genome sequences are currently known and predictive programs for gene structure have improved in recent years. Here, we reexamined the predicted signal peptide in this protein using the latest software and attempted to confirm its presence.

Materials and Methods

Bacterial strain and growth condition

P. gingivalis FDC381 was anaerobically grown at 37 °C in trypticase soy broth supplemented with phytone peptone (5 mg/ml), yeast extract (5 mg/ml), NaCl (5 mg/ml), cysteine (0.1%), hemin (5 mg/ml), menadione (0.2 mg/ml) and kanamycin (50 µg/ml). Recombinant *Escherichia coli* MD401 containing the *hbp35* gene (8, 15) was grown in Luria-Bertani broth in the presence of ampicillin (50 µg/ml).

Cell fractionation

After centrifugation of bacterial suspension, MD401 cells were suspended in Dulbecco's phosphate-buffered saline without Ca²⁺ and Mg²⁺ (PBS⁻) and were disrupted by sonication (crude extract). A periplasmic fraction was prepared by cold osmotic-shock treatment (18, 19).

Preparation of anti-rHBP35 antibody

Antiserum against recombinant HBP35 (rHBP35) was obtained by immunizing rabbits via injection of purified rHBP35 with Freund's incomplete adjuvant, at several sites on the back once a week for 4 weeks (15). An affinity-purified rHBP35 IgG fraction was purified from anti-rHBP35 rabbit serum using a diethyl-aminoethyl cellulose column (DE52; Whatman Ltd., Maidstone, UK) and an immuno-affinity column (Glycosyl-hardgel; ICN ImmunoBiologicals, Lisle, IL, USA). The antibody was suspended with 1% BSA in PBS⁻.

SDS-PAGE and Western blot analysis

Crude extract and the periplasmic fraction of the

MD401 cells were denatured by boiling for 5 min in SDS-PAGE sample buffer. Proteins were separated by SDS-PAGE on a 12% resolving gel in a MINI-PROTEIN II Cell (Bio-Rad Laboratories Inc., Tokyo, Japan), and protein bands were visualized by staining with Coomassie Brilliant blue R-250. For Western blot analysis, samples were transferred from the SDS-PAGE gels to PVDF membranes using a semi-dry TRANS-BLOT transfer apparatus (Bio-Rad). After incubation for 30 min at room temperature in blocking reagent (5% dried skim milk in PBS⁻ and 0.1% Tween 20), the membrane was probed with primary antibodies and HRP-conjugated anti-rabbit IgG as secondary antibodies. Membranes were washed and developed with color-developing reagent (4-methoxy-1-naphthol; Bio-Rad) in hydrogen peroxide. Anti-rHBP35 antibody of *P. gingivalis* FDC381 was used as primary antibody.

Amino-terminal sequence analysis

The low molecular weight HBP35 protein was purified by reversed-phase HPLC from the crude extract of MD401 cells. After dissolving in 1% trifluoroacetic acid, amino acids were analyzed by automated Edman degradation in a 470A gas-phase sequencer (Applied Biosystems Inc., Foster City, CA, USA).

Computational analyses

Amino acid sequence and homology of the HBP35 protein were analyzed using GENETYX-MAC computer software (version 16.0; GENETYX Co., Tokyo, Japan).

Signal peptide prediction was performed using SignalP 3.0 server (<http://www.cbs.dtu.dk/services/SignalP/>). SignalP 3.0 server predicts the presence and location of signal peptide cleavage sites in amino acid sequences from different organisms: Gram-positive prokaryotes; Gram-negative prokaryotes; and eukaryotes. The method incorporates the prediction of cleavage sites and signal peptide/non-signal peptide prediction based on a combination of several artificial neural networks and hidden Markov models. Analysis was performed under the following

conditions: Organism group, Gram-negative bacteria; Method, neural networks and hidden Markov models; Truncate each sequence to max, 70 residues (20-22).

For prediction of subcellular protein localization, SOSUI-GramN (http://bp.nuap.nagoya-u.ac.jp/sosui/sosuigramn/sosuigramn_submit.html) (22) and PSORTb (ver. 3.0; <http://www.psort.org/psortb/>) (24) were used.

PSORTb consists of multiple analytical modules, each of which analyzes one biological feature known to influence or be characteristic of subcellular localization. To analyze Gram-negative organisms, CMSVM- (for identification of cytoplasmic membrane proteins), CytoSVM- (for identification of cytoplasmic proteins), ECSVM- (for identification of extracellular proteins), ModHMM- (for prediction of transmembrane helices within the sequence), Motif- (to search the sequence for Gram-negative motifs indicative of specific localization sites), OMPMotif- (to search the sequence for Gram-negative outer membrane protein motifs), OMSVM- (for identification of outer membrane proteins), PPSVM- (for identification of periplasmic proteins), Profile- (to search the sequence for Gram-negative profiles indicative of a specific localization site), SCL-BLAST- & SCL-BLASTe- (BLASTP search against the Gram-negative subset of the current PSORTdb dataset) and Signal- (to search the sequence for the presence of a Gram-negative cleavable N-terminal signal peptide) were used for multiple analytical modules. Possible localization sites are predicted in the cytoplasm, cytoplasmic membrane, periplasm, outer membrane and extracellular space.

Immunostaining

P. gingivalis cells were grown in diffusate broth. After centrifugation at $10,000\times g$ for 30 min, the resulting pellet was washed three times with PBS⁻. The pellet was resuspended in 2.5% (v/v) glutaraldehyde (pH 7.2), and was incubated at 4 °C for 2 h. After washing with same buffer at 4 °C for 10 min, cells were dehydrated through a graded ethanol series (50, 60, 70, 80, 90, 95 and 100% for 1 h each) at -20 °C, followed by infiltration with ethanol-Lowicryl K4M (PolyScience Inc., Washington, PA, USA) at (1:1) and (1:2) for 1 h each at -20 °C and embedding in Lowicryl K4M. After polymerization under UV irradiation (365 nm) overnight at -20 °C and for 2 days at room temperature, ultrathin sections were mounted on 200 mesh nickel grids.

Sections were incubated with 1% (v/v) goat serum and 0.1% (v/v) BSA at room temperature for 30 min, followed by anti-rHBP35 affinity-purified antibody at room temperature for 2 h. Sections were washed three times with PBS⁻ at room temperature for 10 min, and were incubated with a protein A-gold (15 nm) complex at room temperature for 1 h. After washing with PBS⁻ for 10 min, and washing twice with distilled water for 10 min, dried sections were stained with uranyl acetate and lead citrate. Specimens were observed under an electron microscope (JEM 200CX; JEOL Ltd., Tokyo, Japan).

Nucleic acid and predicted protein sequence of *hbp35*

The nucleic acid sequence of *hbp35* is deposited in GenBank under the accession number AB059658, and the predicted amino acid sequence (344 amino acid residues) is shown in Fig. 1.

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1 MKRLLLSAAI LSSMALFNVN AQELKTSADM KGSFKKNVVL EVFTAEWCGY
51 CPGGKERIAK AIEMLDDEYK ERVFQTFVHY NDGISKKWPR VGQLFIALDQ
101 TLGIPGFPTF SVCRMEKKGE NLSIGAPIAI KNKIMKGFGD GTAPAEVNLK
151 LTKGATPEDV CTATFTGKVD ADLIGKPLML TAYVLKNNMK PINPQNGAGD
201 GYLHQHTVLM ILSTDVKGDA LNIAADGSFT IKKEFKLDGF EIKDTDVLAF
251 VHHPSNAEN HSIINAGQES LDKAEPATE QIVATPSVKA YVQNGKIVVE
301 EEYSKMEVFN ATGQLVKNES LVPGVYVRI TANGVMHFLK VLVP

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Fig. 1. Predicted amino acid sequence of *hbp35* gene encoding a 40-kDa outer membrane protein in *P. gingivalis*. DNA sequence is deposited in GenBank under the accession number AB059658. The partial amino acid sequence at the N-terminus of the low molecular weight HBP35 protein is underlined.

Results

Signal peptide analysis

Fig.2 shows the signal peptides predicted by SignalP 3.0 server. The highest S-score is observed at the 9th amino acid residue, and lower scores were seen from the 22nd amino acid, thus suggesting that the length of the signal peptide is 21 residues. The 22nd amino acid shows a markedly higher C-score, which indicates that the mature protein starts at position 22. Y-max is a derivative of the C-score combined with the S-score, resulting in better cleavage site prediction than raw C-score alone. Here, Y-max was seen at the 22nd position. The Y-score in Fig. 2 confirms a very high peak at the 22nd amino acid. Thus, SignalP analysis predicted that the signal peptide region of HBP35 is from the 1st to 21st amino acid residues.

Western blot analysis of MD401 cells was performed with anti-rHBP35 antibody in order to confirm the presence of signal peptides (Fig. 3). The antibody reacted with two protein bands in the crude extract. However, it only reacted with the lower molecular weight band in the periplasmic fraction. These results suggest the presence of HBP35 proteins having two different molecular weights, and that the

Fig. 3. SDS-PAGE and Western blot analysis of *P. gingivalis*. Crude extract (Lane 1) and periplasmic fraction (Lane 2) of *P. gingivalis* cells were separated by gel electrophoresis, and were visualized by staining with Coomassie Brilliant blue R-250 (A). Proteins from SDS-PAGE gel were transferred to a PVDF membrane, and were reacted with anti-rHBP35 antibody and HRP-conjugated anti-rabbit immunoglobulin G. The membrane was developed with color-developing reagent and hydrogen peroxide (B). Numbers at the right indicate the molecular mass of the markers in kDa. Arrows indicate HBP35 protein bands.

Fig. 2. Signal peptide analysis of HBP35 using SignalP 3.0 server. Amino acids from 1st to 70th position of HBP35 are shown in the horizontal axes. C-score indicates the predicted cleavage site. S-score indicates the amino acids that are part of the signal peptide or mature protein. Y-score is a derivative of C-score and S-score.

lower molecular weight protein is mature HBP35 obtained by cleavage of signal peptides.

In order to confirm the presence of signal peptides, the low molecular weight HBP35 was purified from crude extract of MD401 cells by reversed-phase HPLC. The amino acid sequence was then analyzed using a peptide sequencer. The resulting sequence for HBP35 was N-QELKTSADMKG SFKKNVVLE-C at the N-terminus. This was identical to the amino acid sequence of the original HBP35 from the 22nd to 41st residues (Fig. 1). The cleavage site of the signal peptide was thus confirmed to be between 21st and 22nd amino acid residues.

HBP35 protein subcellular localization

For prediction of HBP35 subcellular localization, both SOSUI-GramN and PSORTb were used. SOSUI-GramN suggested that the subcellular location site of the HBP35 was the periplasm, while the localization scores by PSORTb were cytoplasm (0%), cytoplasmic membrane (25%), periplasm (25%), outer membrane (25%) and extracellular space (25%). Both programs suggest that mature HBP35 has non-cytoplasmic localization.

Western-blotting analysis showed that HBP35 protein was located in the periplasmic fraction in recombinant *E. coli*. However, the location of HBP35

in *P. gingivalis* was unclear. Therefore, immunostaining of *P. gingivalis* cells was performed with anti-rHBP35 antibody (Fig. 4). Electron microscopy showed that HBP35 protein was mainly located outside of the *P. gingivalis* cytoplasm.

Discussion

Numerous secreted proteins are important in bacterial pathogenesis and are a primary source of candidates for vaccines and diagnoses. Secretory proteins are generally synthesized with an oligopeptide sequence at the N-terminus called the leader or signal peptide. These peptides direct the protein to a location from the cytoplasm to the periplasmic space of prokaryotic cells; the bulk of exported proteins are dependent on an N-terminal secretion signal and the activity of signal peptidase I.

The signal peptide is recognized by SecA, which transports the signal peptide-containing protein to a membrane-spanning complex. While the signal peptide is inserted into the membrane, the remainder of the protein is translocated across the membrane via the protein complex in an unfolded configuration. As the signal peptide is cleaved off, the mature protein is released to the exterior of the cell. Generally, signal peptides comprise 15 to 30 amino acid residues and contain a highly hydrophobic domain, with the exception of at least one positively charged residue near the methionyl initiation residue.

The N-terminal signal sequence starts with a positively charged region, and is followed by a hydrophobic region and the C-terminal region, which includes the cleavage site (25, 26). In HBP35 protein, the signal peptide sequence, N-MKRLLLSAAILSSM ALFNVNA-C, resulting from initiation at ATG would be 21 residues long. This contains 13 hydrophilic and two positively charged residues near the initiation residue. The peptide sequence has 100% homology with the 35-kDa hemin binding protein of *P. gingivalis* ATCC33277, thioredoxin of *P. gingivalis* W83, and the 35-kDa hemin binding protein of *P. gingivalis* TDC60. All are identical proteins present in different *P. gingivalis* strains. No significant homology was found with other proteins in a search

Fig. 4. Immunoelectron microscopy of *P. gingivalis* cells with anti-rHBP35 antibody. The section was incubated with both anti-rHBP35 affinity-purified antibody and the protein A-gold (15 nm) complex. The section was then stained with uranyl acetate and lead citrate. Bar indicates 0.1 μ m.

of National Center for Biotechnology Information Search database, indicating that the constituent amino acids of the signal peptides have a shared characteristic; however, the amino acid sequence may be unique.

The HBP35 protein is located in bacterial cells and in vesicles released from the outer membrane (8). In addition, the anti-rHBP35 antibody inhibits the adherence of *P. gingivalis* cells, aggregation of erythrocytes and coaggregation with other bacterial cells (10, 12, 15, 16). In this study, Western blot analysis suggested that the HBP35 protein is translocated to the periplasmic space of *P. gingivalis*. The prediction programs for protein localization indicated that mature HBP35 has non-cytoplasmic localization, and this was confirmed by electron microscopy. This demonstrates that HBP35 protein is mainly located outside the cytoplasm of *P. gingivalis* cells. These data suggest that HBP35 passes through the inner membrane after cleavage of the signal peptide at the N-terminus, and remains bound to the outer membrane in this bacterium.

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