Human Lactoferrin Binds and Removes the Hemoglobin Receptor Protein of the Periodontopathogen Porphyromonas gingivalis*

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Porphyromonas gingivalis possesses a hemoglobin receptor (HbR) protein on the cell surface as one of the major components of the hemoglobin utilization system in this periodontopathogenic bacterium. HbR is intragenically encoded by the genes of an arginine-specific cysteine proteinase (rgpA), lysine-specific cysteine proteinase (kgp), and a hemagglutinin (hagA). Here, we have demonstrated that human lactoferrin as well as hemoglobin have the abilities to bind purified HbR and have demonstrated that human lactoferrin as well as hemoglobin have the abilities to bind purified HbR and the cell surface of P. gingivalis through HbR. The interaction of lactoferrin with HbR led to the release of HbR from the cell surface of P. gingivalis. This lactoferrin-mediated HbR release was inhibited by the cysteine proteinase inhibitors effective to the cysteine proteinases of P. gingivalis. P. gingivalis could not utilize lactoferrin for its growth as an iron source and, in contrast, lactoferrin inhibited the growth of the bacterium in a rich medium containing hemoglobin as the sole iron source. Lactoferricin B, a 25-amino acid-long peptide located at the N-lobe of bovine lactoferrin, caused the same effects on P. gingivalis cells as human lactoferrin, indicating that the effects of lactoferrin might be attributable to the lactoferricin region. These results suggest that lactoferrin has a bacteriostatic action on P. gingivalis by binding HbR, removing it from the cell surface, and consequently disrupting the iron uptake system from hemoglobin.

Pathogenic bacteria have developed various strategies for acquiring iron from their hosts to support their growth, including production of siderophores to chelate iron ions from their environment and presentation of receptors to acquire iron and heme from iron- and heme-containing proteins such as transferrin, lactoferrin, and hemoglobin (1–3). Porphyromonas gingivalis is a highly proteolytic Gram-negative anaerobic bacterium that is implicated as one of the major causative pathogens for advanced adult periodontitis (4). Heme is required for its growth (5–7). So far it has not been reported that P. gingivalis possesses a hemoglobin receptor (HbR) for acquisition of iron from hemoglobin (9, 10). HbR, a 19-kilodalton protein, was intragenically encoded by an arginine-specific cysteine proteinase (Arg-gingipain, RGP)-encoding gene (rgpA), a lysine-specific cysteine proteinase (Lys-gingipain, KGP)-encoding gene (kgp), and a hemagglutinin (HA)-encoding gene (hagA) (11). Several pieces of evidence show that HbR forms a large proteinase-adhesin complex with RGP, KGP, and HA proteins that are encoded intragenically by these genes (12, 13), and appears to be associated with a lipid A portion of lipopolysaccharide (LPS) (14). P. gingivalis has another RGP-encoding gene (rgpB); however, it does not encode HbR or HA (15). We found in a previous study (11) that a purified HbR protein has the ability to bind hemoglobin. In addition, analysis with the rgpA kgp hagA and rgpA rgpB kgp triple mutants that failed to express HbR on their cell surfaces revealed that HbR was dispensable for P. gingivalis cells to bind hemoglobin (16). These findings indicate that HbR is a major component for the iron acquisition/utilization system from hemoglobin in P. gingivalis.

Lactoferrin is an iron-binding, acute-phase protein found in saliva, milk, and other exocrine secretions (17). Lactoferrin is secreted from activated neutrophiles and may mediate the amplification of the inflammatory response, phagocytosis, and regulation of myelopoiesis (18–22). Several mechanisms for the antimicrobial action of lactoferrin have been proposed. First, lactoferrin has the ability to chelate iron, which results in inhibition of bacterial growth (23). Second, it has been found that a peptide named lactoferricin, which is liberated from lactoferrin by degradation with gastric pepsin, has a bactericidal action. This looped peptide of the N-lobe of the exposed surface of a lactoferrin molecule is distinct from the iron binding region (24–26). The mechanism of bactericidal action of this cationic peptide may be elucidated to be, as in other antimicrobial peptides such as cecropins and magainins, that the peptide interacts with negatively charged divalent cation binding sites on bacterial cell surfaces such as LPS, disrupts these sites, and leads to the uptake of peptides across the outer membrane. The affected membrane forms a channel, resulting in leakage of cytoplasmic molecules and cell death (27, 28). Third, it has been demonstrated recently that lactoferrin specifically inactivates colonization factors of Hemophilus influenzae and attenuates the pathogenic potential of this bacterium (29).

The concentration of lactoferrin rises to as high as 20 μM in the gingival crevicular fluids of patients with localized juvenile periodontitis, gingivitis, and adult periodontitis (30, 31). P. gingivalis cells have the ability to adsorb lactoferrin (32), but it is not yet known what molecule(s) on the cell surface is(are) responsible for this adsorption. In addition, it has not been determined whether P. gingivalis cells utilize iron from lactoferrin or whether lactoferrin attenuates their growth as an antimicrobial agent.

In the present study, We found that human lactoferrin bound to HbR and removed it from the cell surface of P. gingivalis and...
that the lactoferrin region of lactoferrin and the N-terminal anion-rich region of HbR might be responsible for the interaction of lactoferrin with HbR. We also found that lactoferrin could not support the growth of *P. gingivalis* as an iron source. In contrast, the growth of *P. gingivalis* was inhibited by lactoferrin when it was grown in a rich medium containing hemoglobin as the sole iron source.

**EXPERIMENTAL PROCEDURES**

**Strains, Media, and Conditions for Cell Growth—*P. gingivalis* strains used here are listed in Table I. *P. gingivalis* cells were grown anaerobically (10% CO2, 10% H2, and 80% N2) in enriched brain heart infusion (BHI) medium and on enriched tryptic soy agar (11). Hemin (Wako, Japan) was used routinely as the iron source at 7.7 μM. For hemoglobin-containing enriched BHI medium, human hemoglobin was added to the medium at 50 nM.

**Proteins and Proteinase Inhibitors—** Human hemoglobin, human globin, human iron-saturated lactoferrin, and human milk lactoferrin (6% iron-saturated) were purchased from Sigma. Bovine lactoferrin (lactoferrin B) (24) was a gift from Nutritional Science Laboratory, Morinaga Milk Industry, Zama, Japan. Proteinase inhibitors, Nα-p-tosyl-l-lysine chloromethyl ketone (TLCK), and leupeptin and phenylmethylsulfonyl fluoride (PMSF) were purchased from Sigma, and leupeptin and phenylmethylsulfonyl fluoride (PMSF) were purchased from Peptide Institute (Minch, Japan) and Nacalai Tesque (Kyoto, Japan), respectively.

**Surface Plasmon Resonance Analysis—** HbR protein was overproduced in *Escherichia coli* BL21(DE3) harboring pKD349, which contains the HbR domain region DNA, and purified as described previously (11). The interactions of HbR with several proteins were determined by surface plasmon resonance analysis using a BIAcore instrument (BIAcore 2000; Amersham Pharmacia Biotech). HbR was cross-linked to the dextran matrix of the sensor chip (CM5; Amersham Pharmacia Biotech). The dextran matrix was activated with N-hydroxysuccinimide (Sigma) and N-ethyl N-3-(dimethylamino)propyl)-carbodiimide hydrochloride (Sigma). HbR (100 μg/ml) was injected at a flow rate of 5 μl/min for 5 min in 10 μl acetic acid buffer (pH 3.5), and unreacted sites in the matrix were blocked with 1 M ethanolamine hydrochloride (pH 8.5) (Sigma). The running buffers used in acidic and neutral conditions were 50 mM acetate buffer (pH 5.5) and 50 mM Tris-HCl (pH 7.5), respectively. Several proteins in the running buffers were passed over the chip at a rate of 10 μl/min for 4 min followed by a wash of the buffers alone for 8 min. Their binding to HbR was measured in real time by changes in optical properties near the sensor surface. The sensor surfaces were regenerated between assays by a 5-min injection of 50 mM Tris-HCl buffer (pH 9.0) for removal of hemoglobin and by a 5-min injection of 0.2 M EDTA (pH 11.0) for removal of lactoferrin. All BIAcore analyses were carried out at 25 °C. The estimation of the rate constants of dissociation (k_d) and association (k_a) were performed using a software package (BIAevaluation 2.1, Amersham Pharmacia Biotech) in which the data from the sensograms were calculated for k_d using the following equation: ln(R_o/R) = k_d t + t_0, the response unit (RU) of signal at the time of the initial stage of the dissociation (t_0); R_o, RU at time t; t_0, t, k_d was calculated by the equation: dRU/dt = k_d C; dRU/dt, change of RU by time; C, concentration of lactoferrin. The dissociation constant (K_d) of protein-protein binding was calculated from the following equation: K_d = K_d/K_a.

**Dot Blot Analysis—** *P. gingivalis* cells grown in enriched BHI medium for 48 h were harvested by centrifugation at 9000 × g for 5 min, washed with phosphate-buffered saline (PBS) (pH 7.4), and resuspended in the original volume of PBS. The washed cells (5 μl) were blotted directly onto a nitrocellulose membrane (Bio-Rad). This membrane was then blocked with PBS containing 0.5% skim milk (Difco) and 0.2% Tween 20 for 1 h and probed for 1 h with diluted (1:1000) human milk lactoferrin that had been conjugated with horseradish peroxidase (HRP, Sigma) according to Kishore et al. (33). Unbound HRP-conjugated lactoferrin was removed by washing with PBS four times for 5 min each. HRP-conjugated lactoferrin was stained using an HRP color substrate (4-chloro-1-napthol, Sigma). The running buffers used in acidic and neutral conditions were 50 mM acetate buffer (pH 3.5), and 137 mM NaCl, 0.1% Tween 20) for 1 h and washed with PBS-Tween three times for 10 min each. The membrane was then incubated in PBS-Tween containing 10% glycerol and 10 μg/ml HbR or a mutant HbR for 1 h. After being washed with PBS-Tween three times for 10 min each, the membrane was incubated in PBS-Tween containing anti-HbR rabbit antibody (1:1000) for 1 h, washed with PBS-Tween three times for 10 min each, and incubated in PBS-Tween containing HRP-conjugated anti-rabbit IgG (1:2, 000) (Santa Cruz Biotechnology) for 30 min, and was washed again three times with PBS-Tween. All the procedures were done at room temperature. The peroxidase activity was detected by exposure of the membrane to x-ray film using ECL detection systems (Amersham Pharmacia Biotech).

**Removal of HbR from *P. gingivalis* Cells by Lactoferrin**

For analysis of lactoferrin-HbR interaction on a membrane, human milk lactoferrin (5 μg) was spotted onto a nitrocellulose membrane and left to dry. The membrane was blocked with 2% bovine serum albumin in Tris-buffered saline (TBS)-Tween (20 mM Tris-HCl (pH 7.5), 137 mM NaCl, 0.1% Tween 20) for 1 h and washed with PBS-Tween three times for 10 min each. The membrane was then incubated in PBS-Tween containing 10% glycerol and 10 μg/ml HbR or a mutant HbR for 1 h. After being washed with PBS-Tween three times for 10 min each, the membrane was incubated in PBS-Tween containing anti-HbR rabbit antibody (1:1000) for 1 h, washed with PBS-Tween three times for 10 min each, and incubated in PBS-Tween containing HRP-conjugated anti-rabbit IgG (1:2, 000) (Santa Cruz Biotechnology) for 30 min, and was washed again three times with PBS-Tween. All the procedures were done at room temperature. The peroxidase activity was detected by exposure of the membrane to x-ray film using ECL detection systems (Amersham Pharmacia Biotech).

**Treatment of P. gingivalis Cells with Lactoferrin and Immunodetection of HbR—** *P. gingivalis* cells in 48 h culture were harvested by centrifugation at 9000 × g for 5 min, washed with PBS twice, and then resuspended in 0.5 volume of PBS. Human milk lactoferrin or lactoferrin B was added to a series of cell suspensions to final concentrations of 0, 2.4, 12.5, and 25 μM. These mixtures were kept at 37 °C for 90 min. After treatment, TLC and leupeptin were added at 100 μg/ml, and the mixtures were centrifuged at 9000 × g for 5 min to separate supernatants and cell pellets. The cell precipitates were then resuspended in an equal volume of PBS. These supernatants and cell suspensions were boiled after being mixed with one-sixth volume of the sample buffer for SDS-PAGE. Samples (10 μl) were subjected to SDS-PAGE (15% gel) and electrotransferred to a nitrocellulose membrane. Membranes were blocked by PBS containing 5% skim milk for 1 h, washed twice with PBS-Tween, and incubated in PBS-Tween containing anti-HbR rabbit antibody (1:1000) or anti-timulin rabbit antiserum (1:2000) for 1 h. Membranes were washed with PBS-Tween three times for 5 min each, incubated in PBS-Tween containing the HRP-conjugated anti-rabbit immunoglobulin antiserum (1:2000) (Santa Cruz) for another 1 h, and then washed with PBS-Tween three times. Detection of the peroxidase activity was the same as described above.

**Construction of an *E. coli* strain Overexpressing a Truncated Mutant HbR**

The nucleotide sequence encoding the amino acid sequence of HbR from the 28th residue (Gly-28) to the last residue (Lys-135) was amplified from pKD349 DNA by PCR with oligonucleotide primers (upper primer, 5’-GACATATGGTAGCGGCGGTTGC-3’, lower primer, 5’-CTGAGATCTGCTTGCGGCGGTTGC-3’). The PCR product was cloned into pCR2.1 (Invitrogen), and further subcloned into pET11a at the NdeI and BamHI regions, resulting in pKD355. *E. coli* BL21(DE3) harboring pKD355 produced a truncated mutant HbR (HbR(del27)) losing the first 27 N-terminal amino acid residues.

**Purification of HbR(del27)**

For purification of HbR(del27), *E. coli* BL21(DE3) harboring pKD355 was grown to an absorbance at 540 nm of 0.5 in 300 ml of L broth containing 50 μg/ml ampicillin. Isopropyl-β-D-thiogalactopyranoside was added to the culture at a concentration of 0.5 mM and the culture was incubated for another 1 h period to overproduce HbR(del27). The cells were collected by centrifugation at 9000 × g for 5 min, resuspended in 10 ml of Tris buffer (50 mM Tris-HCl, pH 8.0), and disrupted by sonic oscillation. After centrifugation at 9000 × g for 15 min, the precipitate was washed twice with Tris buffer, dissolved in Tris buffer containing 6 M guanidine-HCl, and centrifuged at 9000 × g for 10 min to remove insoluble materials (HbR(del27)) was formed as an inclusion body. The supernatant was mixed with the same volume of glyceral and dialyzed against Tris buffer containing 75% glycerol at 4 °C overnight. The dialyzed solution was diluted with Tris buffer to a glyceral concentration of 10%.

**Silver Staining of LPS**

LPS samples were prepared according to the method of Perez and Blaser (34). After the samples were subjected to SDS-PAGE, LPS samples were prepared according to the method of Hitchcock (35).
in an equal volume of PBS. After 5 m
mutant cells. Cells were anaerobically grown in enriched BHI
givalis
Dots 1–4
brane was incubated with hemoglobin at various concentrations for 1 h.
KDP133,
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30004
In a previous study (16), we found that
P. gingivalis
hemoglobin could bind to the cell surfaces of
P. gingivalis
kgp
ATCC 33277 (wild type), KDP129 (rgpA kgpB), and KDP134 (rgpA kgp). On the other hand, neither KDP136
(rgpA rgpB kgp) nor KDP137 (rgpA kgp hagA) had the ability to bind hemoglobin. These previous results were consistent with the presence or absence of HbR on the cell surfaces. Together with the finding that HbR has the ability to bind hemoglobin, they indicate that HbR is the most important receptor for hemoglobin in P. gingivalis. Here, the dot blot assay with HRP-conjugated lactoferrin showed that the intact cells of the wild type, kgp, and rgpA rgpB strains could adsorb lactoferrin, whereas the rgpA kgp, rgpA rgpB kgp, and rgpA kgp hagA strains could not (Fig. 1a). The lactoferrin adsorption was very similar to hemoglobin adsorption in the strains used except for the rgpA kgp mutant, indicating that lactoferrin adsorption by P. gingivalis cells may be attributable to the presence of HbR on the cell surface.

Ability of Lactoferrin to Bind HbR—We determined the ability of lactoferrin to bind HbR by surface plasmon resonance analysis with an HbR-immobilized sensor chip (Table II). The dissociation coefficients between lactoferrin and HbR were calculated from sensograms using a series of concentrations of samples. Interestingly, lactoferrin strongly bound HbR at both pH 5.5 and 7.5, whereas hemoglobin showed strong HbR binding only at acidic pH in this cell-free system as previously found (11). Both iron-saturated and 6% iron-saturated lactoferrin could bind HbR. In addition, globin was found to bind HbR, implying that the interaction of HbR with hemoglobin may occur at protein proportions rather than heme. Neither bovine serum albumin nor human ferritin could bind HbR (data not shown). Free HbR efficiently inhibited the binding between lactoferrin and the immobilized HbR in a concentration-dependent manner (data not shown).

Inhibition of the Binding of Hemoglobin to HbR by Lactoferrin—To clarify the interaction between lactoferrin and hemoglobin in the binding to HbR, we tested the binding of hemoglobin to the HbR-immobilized sensor chip on which lactoferrin had been passed three times in advance. The binding of hemoglobin to HbR was completely diminished when lactoferrin was prebound to the chip at a saturation level (Fig. 2). A similar result was obtained in the binding of lactoferrin to a hemoglobin-prebound HbR-immobilized chip (data not shown). To determine whether this inhibition takes place on the surfaces of intact P. gingivalis cells, we adopted the dot blot assay. Thus, we examined the binding of HRP-conjugated lactoferrin to intact P. gingivalis cells that had been blotted on a nitrocellulose membrane and preincubated with various concentrations of hemoglobin (Fig. 1b). Adsorption of lactoferrin to the cells was inhibited by preincubation with hemoglobin in a concentration-dependent manner. These results indicate that lactoferrin and hemoglobin competitively bind HbR on P. gingivalis cell surfaces.

Lactoferrin-mediated Growth Suppression of P. gingivalis Cells in Hemoglobin-containing Medium—P. gingivalis wild type strain was able to grow in enriched BHI medium containing 50 μM hemin as well as in medium containing 7.7 μM hemin (data not shown). To clarify the effect of lactoferrin on the growth of the P. gingivalis cells, the cells were incubated in the hemoglobin- or hemin-containing medium to which lactoferrin was added at various concentrations. Cell growth was significantly retarded in the hemoglobin-containing medium supplemented with lactoferrin at 13.6 and 24.8 μM (Fig. 3a). On the other hand, lactoferrin did not suppress the growth of P. gingivalis cells in the hemin-containing medium even at 24.8 μM (Fig. 3b). The rgpA kgp mutant showed no lactoferrin binding but did show hemoglobin binding (Fig. 1c, Ref. 16). Interestingly, the lactoferrin-mediated growth suppression of the rgpA kgp mutant in the hemoglobin-containing medium was weaker than that of the wild type parent (Fig. 3c). These results indicate that the growth retardation by lactoferrin may be due to the ability of lactoferrin to access HbR, to interfere

![Table II](https://example.com/table.png)

| Human globin | 4.63 × 10⁻⁹ | 5.20 × 10⁻⁹ |
| Human iron-saturated lactoferrin | 4.36 × 10⁻⁹ | 8.27 × 10⁻¹⁰ |
| Human milk lactoferrin (6% iron-saturated) | 1.12 × 10⁻⁹ | 2.97 × 10⁻¹⁰ |

* n.d., could not be determined because of low affinity.
Observed in enriched BHI medium containing iron-saturated lactoferrin or hemin as the sole iron source. No growth was achieved when ATCC 33277 were incubated in enriched BHI medium containing lactoferrin as an iron source, iron-deprived cells of the wild type strain were fully grown in enriched BHI medium containing hemoglobin (50 μM) and various concentrations of lactoferrin, indicating that lactoferrin cannot utilize lactoferrin as an iron source (data not shown).

To determine whether \( P. \) gingivalis cells can utilize lactoferrin as an iron source, iron-deprived cells of the wild type strain were incubated in enriched BHI medium containing lactoferrin or hemin as the sole iron source. No growth was observed in enriched BHI medium containing iron-saturated lactoferrin (6.2 μM) up to 90 h after incubation, whereas the cells were fully grown in enriched BHI medium containing hemin, indicating that \( P. \) gingivalis cannot utilize lactoferrin as an iron source (data not shown).

**Release of HbR from \( P. \) gingivalis Cell Surfaces by Lactoferrin**—To determine whether lactoferrin affects \( P. \) gingivalis cell surfaces, we treated \( P. \) gingivalis cells with lactoferrin at the same concentrations as those of lactoferrin in the gingival crevicular fluids of patients with acute inflammation (up to 24.8 μM) and healthy subjects (about 6.2 μM) (30, 31). We separated the lactoferrin-containing cell suspension into the cell precipitates and supernatants by centrifugation and examined the presence of LPS, fimbriulin, and HbR in each portion. Lactoferrin did not release LPS in the conditions used in these experiments (Fig. 4a). No change was seen in the amounts of fimbriulin in the supernatants before and after lactoferrin treatment (Fig. 4b). However, HbR appeared in the supernatants after lactoferrin treatment in a concentration-dependent manner (Fig. 4c). In contrast, no HbR was released when \( P. \) gingivalis cells were treated with hemoglobin even at a high concentration (Fig. 4d). These results indicate that HbR is released specifically from the cell surfaces by the lactoferrin treatment. The lactoferrin-mediated HbR release was effectively inhibited by the addition of iodoacetamide and a mixture of TLCK and leupeptin but not by the addition of PMSF, implying that RGP and/or KGP proteinase domain proteins may be involved in this HbR release (Fig. 4e). The HA proteins, which were also encoded by rrgA, kgp, and hagA as intragenic domain proteins, were not released from the cell surface by lactoferrin treatment (Fig. 4f). The lactoferrin treatment caused no change in viability of \( P. \) gingivalis (data not shown).

HbR is intragenically encoded by rrgA, kgp, and hagA. To determine from which proteins the HbR released by lactoferrin is derived, we examined various mutants for lactoferrin-mediated HbR release (Fig. 5). The rrgA rrgB double mutant and the kgp mutant showed the lactoferrin-mediated HbR release as well as the wild type parent, whereas the rrgA kgp double mutant showed no HbR release. Interestingly, the lactoferrin-mediated HbR release was suppressed by leupeptin and TLCK in the rrgA rrgB mutant but not in the kgp mutant.
The membrane was immunostained with anti-HbR antiserum. The separated proteins in the gel were transferred to a nitrocellulose membrane. The membrane was immunostained with anti-HbR antiserum. Lanes: 1, lactoferrin, 0 μM; 2, lactoferrin, 13.6 μM; 3, lactoferrin, 24.8 μM; 4, lactoferrin, 24.8 μM with leupeptin, 100 μM and TLCK, 100 μM; 5, lactoferrin, 24.8 μM with PMSF, 7.5 mM. Lane 6 contains the cell extracts of those strains with no treatment.

Growth Retardation and HbR Release by Lactoferricin B—Pepsin-digested lactoferrin, as well as intact lactoferrin, caused the release of HbR from the cell surface, whereas trypsin digestion abolished the ability of lactoferrin to release HbR (data not shown). Because these results suggested the possibility that the effects of lactoferrin on P. gingivalis cells might be attributable to the lactoferricin region of a lactoferrin molecule, we went on to examine whether lactoferricin caused the growth retardation and HbR release. We used bovine lactoferricin (lactoferricin B) in this experiment. Lactoferricin B is a 25-amino acid-long peptide including 8 cationic residues, which is located at the N-lobe of bovine lactoferrin and generated by pepsin hydrolysis (24). Growth retardation of the P. gingivalis wild type strain occurred when lactoferricin B was added to the medium containing hemoglobin at the same molar concentrations as human lactoferrin, but it did not take place in the hemin-containing medium (Fig. 3, c and d). Treatment of the P. gingivalis wild type cells with lactoferricin B caused the HbR release, which was suppressed by the addition of iodoacetamide and a mixture of TLCK and leupeptin but not by the addition of PMSF (Fig. 4e). No release of HA proteins was observed in the lactoferricin treatment (Fig. 4f). No change was seen in the viability of P. gingivalis before and after the treatment. These results with lactoferricin B were consistent with those of the lactoferrin treatment, indicating that the lactoferricin region is responsible for the effects of lactoferrin on P. gingivalis cells.

No Binding of Lactoferrin to a Truncated Mutant HbR—The lactoferricin region has a number of cationic amino acid residues. On the other hand, anionic residues reside preferentially in the N-terminal region of HbR (8 Glu and Asp residues in the first 27 residues). To determine whether the N-terminal region of HbR contributes to the HbR-lactoferrin interaction, we constructed an E. coli overexpressing a truncated mutant HbR (HbR(del27)) in which the first 27 amino acid residues were absent (see "Experimental Procedures"). Dot blot analysis with the purified HbR(del27) revealed that the truncated HbR had no ability to bind lactoferrin, indicating that the N-terminal anion-rich region may interact with the cationic lactoferricin region of lactoferrin (Fig. 6).

Effect of Haptoglobin on the Binding of Hemoglobin to HbR—Haptoglobin has the ability to bind hemoglobin (36) and suppress the growth of P. gingivalis in a medium containing hemoglobin (37). To determine whether this growth suppression by haptoglobin is involved in the interaction of hemoglobin with HbR, the effect of haptoglobin on the binding of hemoglobin to HbR was examined. Haptoglobin inhibited the binding of hemoglobin to HbR as revealed by surface plasmon resonance analysis (Fig. 7).

DISCUSSION
Although P. gingivalis grows well in a medium containing hemin or hemoglobin as an iron source, the concentration of hemoglobin required to support its growth is much lower than that of hemin. The concentrations of hemin and hemoglobin to support 50% of the maximal growth rates are 1–5 μM and 1.7 nm, respectively (37), which implies that the iron uptake/utilization systems from hemin and hemoglobin may have, at least in part, different pathways. In a previous study (11), we presented a hypothetical mechanism of the iron uptake/utilization from erythrocytes. We showed that P. gingivalis cells may adhere to erythrocytes by the HA domain proteins intragenically encoded by rgaA, kgp and hagA. After this adherence, the RGP and/or KGP proteinases may extensively digest surface proteins of erythrocytes, resulting in the release of hemoglobin. Released hemoglobin may then be captured by the HbR proteins and proteolytically degraded to release heme. The heme may then be stored on the surfaces of P. gingivalis cells and utilized by the bacterium. By genetic analysis, HbR was found to be the major hemoglobin receptor protein in P. gingivalis (16). We found that haptoglobin, a natural hemoglobin-binding protein, had the ability to inhibit the interaction of hemoglobin.
with HbR by forming a haptoglobin-hemoglobin complex, revealed by surface plasmon resonance analysis with an HbR-immobilized chip. It was reported that haptoglobin suppresses the growth of _P. gingivalis_ in a medium containing hemoglobin (37). These results strongly suggest that HbR may play a crucial role in the acquisition of iron from hemoglobin in a natural niche for _P. gingivalis_.

In this study, we found that lactoferrin, as well as hemoglobin, bound HbR. Lactoferrin is thought to play a pivotal role in the prevention of infection in the host and its ability to sequester iron from potential pathogens has been regarded as an antimicrobial function (23). The antimicrobial function of lactoferrin may not simply be because of the removal of free iron ions from the environment but may involve the interaction between lactoferrin and cell surfaces of microorganisms (38). Moreover, lactoferrin effectively bound to the cell surface of _P. gingivalis_. The results with the various mutants used, except the _rgpA kgp_ mutant, suggest that lactoferrin binding to the cell surface is attributable to the presence of HbR. The _rgpA kgp_ mutant has HbR on the cell surface. However, the reactivity of the mutant cells to anti-HbR antiserum is significantly weaker than that of the wild type (16). No lactoferrin binding of the _rgpA kgp_ mutant may be explained by the possibility that the circumstances around HbR on the cell surface of the mutant are different from those of HbR derived from the _rgpA_ and _kgp_ genes, and this difference changes the accessibility of lactoferrin to HbR because all HbR in the mutant should come from the _hagA_ gene. HbR shares no similarities with the known lactoferrin-binding proteins of prokaryotes such as LbpA and LbpB of _Neisseria meningitidis_ (39).

We also found that lactoferrin suppressed the growth of _P. gingivalis_ in the medium containing hemoglobin as the sole iron source but not in the medium containing hemin. In several Gram-negative bacteria, lactoferrin damages their outer membranes and releases LPS from the cells (40). In _P. gingivalis_, no significant release of LPS, fimbriae/fimbribin, or the HA proteins was found after lactoferrin treatment. In addition, the treatment produced no change in the viability of _P. gingivalis_. In contrast, lactoferrin removed HbR from _P. gingivalis_ cells. Recently, Qiu et al. (29) found that human milk lactoferrin efficiently extracted IgA1 proteinase preprotein from the cell surface of _H. influenzae_ and degraded Hap adhesin and that PMSF completely suppressed the IgA1 extraction and the Hap degradation. IgA1 proteinase and Hap are produced as large polyproteins comprising four and three domains, respectively (41–43). Because the _rgpA_ and _kgp_ gene products were polyproteins consisting of the proteolytic domain proteins (RGP and KGP), HbR, and HA, we investigated whether the potential proteolytic activity of lactoferrin was involved in the release of HbR from the cell surface. The results showed that PMSF did not affect the lactoferrin-mediated HbR release, but interestingly, iodoacetamide and a mixture of leupeptin and TLCK that had the potential to inhibit RGP and KGP proteinases did inhibit the HbR release. HbR makes a complex with proteinase domains and HA domains, and these domains are covalently bound to one another in the early period of growth phase (44). After this period, these domains are separated by RGP and KGP proteinases although the domain proteins remain non-covalently associated (12, 44, 45). Therefore, the inhibitory effect of the proteinase inhibitors on the lactoferrin-mediated HbR release may be due to the inactivation of RGP and KGP proteinase activity that results in inhibition of processing of the polyproteins. However, this explanation seems unlikely because the HbR released into the supernatant appeared to be directly derived from the 19-kDa HbR already processed from the polyprotein, as judged by the finding that the cell-bound HbR before lactoferrin treatment also migrated at a molecular mass of 19 kDa on SDS-PAGE; after treatment the cell-bound 19-kDa HbR decreased in a concentration-dependent fashion. What interaction between lactoferrin and HbR causes the HbR release? We found that lactoferrin did not bind the truncated HbR in which the first 27 amino acid residues, including 8 anionic residues, were deleted and that the growth retardation and HbR release occurred by treatment of lactoferrin B as well as lactoferrin. These results suggest that the cationic lactoferrin region of lactoferrin may interact with the N-terminal anion-rich region of HbR in an electrostatic action, loosen the non-covalent bond between HbR and other components of the complex, and release HbR from the cell surface (Fig. 8). The inhibitory effect of the proteinase inhibitors on the HbR release suggests that a conformational change of the proteinase domain proteins, which can be hindered by the inhibitors, is required in the process of HbR release, although we should reserve the possibility that lactoferrin directly interacts with the proteolytic domain proteins or other components of the complex in addition to HbR. In this context, it is noteworthy that proteinase inhibitors such as TLCK and leupeptin suppress hemagglutination of _P. gingivalis_ cells, which appears to be attributable to the HA proteins of the complex (46–48).

Growth retardation of _P. gingivalis_ by lactoferrin depended on the concentration of lactoferrin. However, after prolonged incubation, _P. gingivalis_ cells grew up to a maximal level even in the medium supplemented with large amounts of lactoferrin, probably because lactoferrin was gradually degraded by proteinases secreted from _P. gingivalis_ and lost its bacteriostatic action. The lactoferrin-mediated growth suppression was not observed in the medium containing hemin instead of hemoglobin. Therefore, the retardation in growth of _P. gingivalis_ may be a consequence of the specific interaction of lactoferrin with HbR, which is the major hemoglobin receptor of the bacterium. These results suggest that lactoferrin may attenuate the infection of _P. gingivalis_ by selectively removing HbR from the cell surface and disrupting the iron acquisition system essential for the _in vivo_ survival of the bacterium and that secreted and
cell-bound RGP and KGP proteins may function as “interceptor missiles” against lactoferrin attack.

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