Extracellular Mycobacterial DNA-binding Protein 1 Participates in Mycobacterium-Lung Epithelial Cell Interaction through Hyaluronic Acid

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Mycobacterium tuberculosis infects not only host macrophages but also nonprofessional phagocytes, such as alveolar epithelial cells. Glycosaminoglycans (GAGs) are considered as the component of mycobacterial adherence to epithelial cells. Here we show that extracellularly occurring mycobacterial DNA-binding protein 1 (MDP1) promotes mycobacterial infection to A549 human lung epithelial cells through hyaluronic acid (HA). Both surface plasmon resonance analysis and enzyme-linked immunosorbent assay revealed that MDP1 bound to HA, heparin, and chondroitin sulfate. Utilizing synthetic peptides, we next defined heparin-binding site of 20 amino acids from 31 to 50 of MDP1, which is responsible for the specific DNA-binding site of MDP1. MDP1 bound to A549 cells, and exogenous DNA and HA interfered with the interaction. The binding was also abolished by treatment of A549 cells with hyaluronidase, suggesting that HA participates in the MDP1-A549 cell interaction. Adherence of bacillus Calmette-Guérin (BCG) and M. tuberculosis to A549 cells was inhibited by addition of HA, DNA, and anti-MDP1 antibody, showing that MDP1 participates in the interaction between mycobacteria-alveolar epithelial cells. Simultaneous treatment of intratracheal BCG-infected mice with HA reduced the growth of BCG in vivo. Taken together, these results suggest that HA participates in Mycobacterium-lung epithelium interaction and has potential for therapeutic and prophylactic interventions in mycobacterial infection.

Attachment of microbial pathogens to host cells is a critical event to develop mucosal infection (1). Mycobacterium tuberculosis persistently infects 32% of the world human population and is responsible for around 1.7 million deaths attributable to a single infectious pathogen each year (2). M. tuberculosis is transmitted by airborne particles and is deposited in a terminal alveolus, where the bacteria are phagocytosed by alveolar macrophages or invade into nonprofessional phagocytic cells such as epithelial pneumocytes (3, 4). Nonprofessional phagocytic cells are presumed to be hiding places of M. tuberculosis in persistent infection, because M. tuberculosis DNA can be frequently detected in type II alveolar epithelial cells and fibroblasts of the lung derived from tuberculin skin test-positive healthy individuals (5). However, the precise mechanism of the interaction between lung epithelial pneumocytes and mycobacteria remains unknown. Better understanding of the interaction is important for developing therapeutic/prophylactic strategies against tuberculosis.

Carbohydrates, such as glycosaminoglycans (GAGs), are thought to be receptors in the process of mycobacterial infection to nonprofessional phagocytic cells (6). GAGs contain hyaluronic acid (HA), heparin, heparan sulfate, and chondroitin sulfates (7). Bacterial pathogens possess adhesion molecule, called adhesin to interact with host tissues. Heparin-binding hemagglutinin (HBHA) binds to GAGs and is identified as a mycobacterial adhesin in the mycobacterium-epithelial cell interaction (8). When bacillus Calmette-Guérin (BCG) or M. tuberculosis lacking HBHA was instilled into mice through the intranasal route, delay of extrapulmonary dissemination was observed (8). Thus interaction with epithelia is thought to be crucial for dissemination and to dominate the disease outcome. Besides HBHA, M. tuberculosis has another possible adhesin, designated mycobacterial cell entry protein 1 (Mcep1), of which DNA fragmentation confers on Escherichia coli an ability to invade into nonphagocytic HeLa cells (9), although its precise role as an adhesin remains unclear (10). It is likely that myco-

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** The abbreviations used are: GAG, glycosaminoglycan; aa, amino acid; BCG, bacillus Calmette-Guérin; BSA, bovine serum albumin; fluos, 5(6)-carboxyfluorescein-N-hydroxysuccinimidyl ester; HA, hyaluronic acid; HBHA, heparin-binding hemagglutinin; GFP, green fluorescent protein; LBP, laminin-binding protein; MDP1, mycobacterial DNA-binding protein 1; PBS, pH 7.4, synthetic peptide corresponding to the amino acid sequence of MDP1 at the 31–50 position; RU, resonance units; SPR, surface plasmon resonance; CBB, Coomasie Brilliant Blue; CHO, Chinese hamster ovary cells; CFU, colony-forming units.
bacteria utilize multiple adhesins to promote attachment to nonprofessional phagocytes.

A number of nonspecific DNA-binding proteins are found in association with bacterial chromosomes and are called histone-like proteins. Mycobacterial DNA-binding protein 1 (MDP1) has a wide range of binding activities to genomic DNA through guanine and cytosine and is an abundant structural protein, like proteins. Mycobacterial DNA-binding protein 1 (MDP1) association with bacterial chromosomes and are called histone-like proteins. Bacteria utilize multiple adhesins to promote attachment to bacteria to lung epithelial cells.

Another interesting feature of this molecule is localization. MDP1 exits not only in the cytoplasmic space associated with the 50 S ribosomal subunit and presumably nucleoid but also on the bacterial surface (11, 15). Nuclear protein is localized in the cytoplasm, and its primitive roles are to compact the genome and control gene expression. However, some nuclear proteins are expressed on the cell surface and play alternative roles. For example, eukaryotic histone H1 on the cell membrane is identified as a receptor for thyroglobulin that is the precursor of thyroid hormones (16). High mobility group 1 protein is a eukaryotic nuclear protein associated with chromatin but also secreted in the extracellular milieu (17). Outside the cell, high mobility group 1 binds to the receptor for advanced glycation end products and then stimulates the cell damage signal. This signal eventually triggers inflammation for clearance of necrotic cells (18–20). Mycobacterium leprae produces LBP, which is a homologue of MDP1, although it lacks DNA-binding activity (15). Instead of DNA-binding activity, LBP interacts with laminin-2 and is considered to be involved in M. leprae invasion into Schwann cells of the peripheral nervous system (15).

The aim of this study was to clarify the role of surface-exposed MDP1 in the interaction between mycobacteria and lung epithelial cells, because DNA-binding proteins tend to bind GAGs that are thought to be receptors in the process of mycobacterial infection to nonprofessional phagocytes (6, 7). Our results demonstrate that extracellular MDP1 acts as an adhesin by binding to GAGs and mediates mycobacterial adherence to A549 human lung epithelial cells by interaction with hyaluronic acid (HA). Treatment of BCG-infected mice with HA resulted in the reduction of mycobacterial growth in vivo. Taken together, HA may play a key role in adherence of mycobacteria to lung epithelial cells.

**EXPERIMENTAL PROCEDURES**

*Culture Medium and Reagents—*RPMI 1640 media, t-glutamine, and 0.05% trypsin EDTA solution, heparin from porcine intestinal mucosa, HA from human umbilical cord, and heparan sulfate from bovine kidney were purchased from Sigma. Chondroitin sulfate A and C were purchased from Calbiochem. Heparinase from porcine intestinal mucosa, and hyaluronidase from Streptomyces hyalurolyticus, heparinase 1 from Flavobacterium heparinum, and chondroitinase ABC from Proteus vulgaris were purchased from Sigma. Polyclonal anti-MDP1 sera were obtained from female rabbit (Japan SLC, Shizuoka, Japan) after multiple injections of MDP1 emulsified in incomplete Freund’s adjuvant. Polyclonal anti-MDP1 antibodies were obtained by 30% ammonium sulfate precipitation of antisera. The salt was then removed by dialysis in PBS.

*Preparation of Subcellular Fractions of BCG—*To obtain subcellular fractions from BCG, all the following procedures were carried out at 4°C. Ten grams of BCG was disrupted in cold PBS with a Bioforce UCD-200T sonicator (Toyo, Tokyo, Japan), and the suspension was centrifuged at 3,000 × g for 5 min to remove unbroken bacteria. The supernatant was centrifuged at 10,000 × g for 10 min. The pellet was rinsed with cold PBS and again centrifuged at 10,000 × g for 10 min. This cell wall-containing pellet was resuspended in 4 ml of cold PBS, and 40% Percoll (Amersham Biosciences) was added. After this, the mixture was centrifuged at 27,000 × g for 1 h to separate cell walls from unbroken cells. The cell wall band was collected and washed twice with PBS and was used as the cell wall fraction. A membrane-ribosome fraction was obtained by centrifugation of the supernatant of the cell wall-containing pellet at 105,000 × g for 2 h. The pellet and the supernatant were used as membrane-ribosome and cytoplasmic fractions, respectively. Culture supernatants of BCG were obtained filtration of culture media of BCG through the membrane filter (pore size of 0.22 μm) and concentrated by 80% of ammonium sulfate precipitation. The salt was then removed by dialysis in PBS. Twenty micrograms of each fraction was fractionated by SDS-PAGE, transferred to the polyvinylidene difluoride membrane, and reacted with polyclonal anti-MDP1 antisera.

*Immunogold Electron Microscopy—*BCG and Mycobacterium smegmatis were grown in Middlebrook 7H9-ADC media at 37°C until A51, and then the bacteria were collected by centrifugation. The bacterial pellet was fixed in 2.5% glutaraldehyde and 2% paraformaldehyde in phosphate buffer, pH 7.4, for 2 h at 4°C and post-fixed in 1% osmium tetroxide in the same buffer for 1 h at 4°C. Specimens were dehydrated in graded ethanol and embedded in Epon-Araldite resin. The thin section was mounted on the nickel grids, treated in 3% H2O2 for 10 min, and washed in water. For blocking the nonspecific binding of antibody to the plastic, the section was preincubated with 3% BSA in PBS for 5 min at room temperature and washed by incubation with rabbit anti-MDP1 antisera diluted in PBS containing 0.1% BSA and 0.05% Tween 20 (PBS-B-T, 1:800) for 2 h at room temperature. The section was then rinsed for 30 min and incubated with protein A conjugated with gold of 10 nm in diameter (EY Laboratories Inc., San Mateo, CA) diluted in PBS-B-T (1:100) for 1 h at room temperature. Finally, the section was rinsed with PBS and distilled water and then briefly stained with uranyl acetate and Reynolds’ lead citrate. The section was analyzed using a H7100 electron microscope (Hitachi Co., Ltd., Tokyo, Japan) operated at 75 kV.

*Proteins Purification—*Native MDP1 and antigen 85B were purified from BCG strain Tokyo as described previously (11, 21). Recombinant MDP1 and HBHA were obtained as follows. Based on the sequence of msd1 and hbha, the oligonucleotide primers, i.e. the forward (5′-CCCATTGAAACAAAGCAGGCTTAGGAC-3′) and reverse (5′-CA-TATGGCTGAAACTCCGAACAT-3′) and the reverse (5′-CCACAGCTTTGCAGCCGGCGAGGCG-3′) and the reverse (5′-GGTGTCCTTGGAATGATTT-3′) primers, were synthesized, respectively. The PCR was carried out by targeting 10 ng of chromosomal DNA derived from BCG. All PCR products were cloned into the pET22 vector (Novagen, Darmstadt, Germany). These plasmids were designated pET22b-MDP1 and pET22b-HBHA, respectively. The DNA sequences of the cloned genes were confirmed by using an ABI 373 automatic DNA sequence (Applied Biosystems, Foster, CA). The pET22b-MDP1 and pET22b-HBHA were introduced into E. coli BL21(DE3)pLys (Invitrogen), and the recombinant bacteria were harbored in LB medium for 50 μg/ml carbenicillin and 34 μg/ml chloramphenicol at 22°C. When an optical density at 600 nm of the culture reached 0.3–0.6, isopropyl-1-thio-β-D-galactopyranoside was added to a final concentration of 0.1 mM, and then the mixture was incubated for additional 7 h. The cells were sonicated as described above, and the supernatant was collected after centrifugation at 12,000 × g for 10 min. After removing the supernatant by filtration through a 0.22-μm membrane filter, it was applied to a 1-ml Hi-Trap chelating column previously charged with 100 mM NiSO4 and equilibrated with 20 mM sodium phosphate, pH 7.4, 10 mM imidazole, and 0.5 M NaCl. After unbound proteins were washed
out, the protein was eluted with the same buffer containing 300 mM imidazole. The fractions containing MDPI were collected and dialyzed against PBS. The purity of proteins was confirmed by SDS-PAGE analysis and stained with Coomassie Brilliant Blue R-250 (CBB) as a single band.

**Heparin-Sepharose Chromatography**—An MDPI-rich, acid-soluble protein fraction was obtained from BCG as described before (11). 200 μg of acid-soluble protein was fractionated by heparin-Sepharose chromatography (Amersham Biosciences), of which bed volume was 1 ml, at room temperature with a linear gradient of NaCl in PBS. Gradients were made in a gradient apparatus filled with 10 ml each of 0.15 and 2 M NaCl solution. A flow rate of 1 ml/min was maintained, and 1 ml of each fraction was collected. The fraction was analyzed by SDS-PAGE. The protein bands were stained with Coomassie brilliant blue.

**Surface Plasma Resonance Measurements**—The interaction between MDPI and GAGs was monitored using SPR using a BLAcore 2000 biosensor (BLAcore AB, Uppsala, Sweden). All binding reactions were performed at 25°C in 10 mM HEPES buffer, pH 7.4, including 150 mM NaCl, 3 mM EDTA, and 0.005% surfactant P20. Proteins and peptide were immobilized on the dextran matrix on the CM5 sensor chip (BLAcore) using an amine coupling kit according to the manufacturer’s instructions (BLAcore). Association and dissociation rate constants were calculated by nonlinear fitting of the primary sensorgram data using BLAevaluation software version 3.0.

**Determination of the Heparin-binding site by ELISA**—20-mer of synthetic peptides corresponding to the amino acid sequence of MDPI (12) were immobilized on the 96-well ELISA plate (Sumitomo, Osaka, Japan) at a concentration of 10 μg/ml in carbonate buffer, pH 9.6, at 4°C overnight. After blocking the wells by 5% BSA in PBS, biotinylated heparin (Sigma) was added at 1 μg/ml in PBS containing 0.05% Tween 20 (PBS-T) to each well and incubated for 1 h at 37°C. After washing unbound heparin, horseradish peroxidase-conjugated streptavidin was added and the mixture was incubated for 1 h at 37°C. After washing free streptavidin, binding was detected by color development with o-phenyldiamine dihydrochloride (Wako, Tokyo, Japan), and ELISA units (optical density) were measured at 492 nm.

**Inhibition of interaction between MDPI and heparin by the synthetic peptide**—A 20-mer of synthetic peptides corresponded to the amino acid sequence of MDPI (12) was immobilized on the 96-well ELISA plate at a concentration of 4 μg/ml in carbonate buffer, pH 9.6, at 4°C overnight. Biotinylated heparin and peptide were premixed in PBS-T for 10 min at 37°C and added into each MDPI-immobilized well. After 30-min incubation at 37°C, unbound heparin was washed out and the level of binding was determined by horseradish peroxidase conjugated with streptavidin as described above.

**Detection of MDPI Binding to GAGs by ELISA and Inhibition Assay by Exogenous DNA**—Genomic DNA of BCG and HA were immobilized on the 96-well ELISA plate (Sumitomo, Osaka, Japan) at a concentration of 10 μg/ml in carbonate buffer, pH 9.6, at 4°C overnight. After blocking the wells by 5% BSA in PBS, biotinylated heparin (Sigma) was added at 1 μg/ml in PBS containing 0.05% Tween 20 (PBS-T) to each well and incubated for 1 h at 37°C. After washing unbound heparin, horseradish peroxidase-conjugated streptavidin was added and the mixture was incubated for 1 h at 37°C. After washing free streptavidin, binding was detected by color development with o-phenyldiamine dihydrochloride (Wako, Tokyo, Japan), and ELISA units (optical density) were measured at 492 nm.

**Protein Labeling—MDPI** was labeled with 5(6)-carboxyfluorescein-N-hydroxysuccinimide ester (fluos, Roche Diagnostics GmbH, Mannheim, Germany). One milligram of MDPI was mixed with 0.237 mg of fluos in 1 ml of PBS for 2 h at room temperature. The molar ratio of fluos to MDPI is 10 to 1. Then, nonreacted fluos was separated by gel filtration on the Sephadex G-25 column (Amersham Biosciences). Similalrly labeled 1 mg of BSA (Sigma) and HBHA were labeled with fluos (molar ratio, 10 to 1). Concentrations of labeled proteins were determined by Bradford’s method (22) using BSA as a standard.

**Protein Binding Assay to A549 Type II Human Lung Epithelial Cells—**A549 cells were suspended at 2 × 10⁵ cells/ml in RPMI 1640 medium containing 10% fetal bovine serum (Serma). 25 mM Hepes, 2 mM L-glutamine, 5.5 mM glucose, 1×10⁻⁵ M 2-mercaptoethanol (complete culture medium), and 1 ml of cell suspensions was dispensed into individual wells of a 24-well plate (BD Biosciences). Plates were incubated at 37°C in a humidified atmosphere at 5% CO₂ for 24 h. The nonadherent cells were poured off, and the residual nonadherent cells were removed by washing with serum-free RPMI 1640 medium twice and refilled with 200 μl of complete culture medium. Fluos-labeled MDPI, BSA, and HBHA was added to a final concentration of 0.5 μM, and the mixture was incubated for 0–5 h. In experiments of enzymatic treatment, epithelial cells were treated with heparinase 1, hyaluronidase, or chondroitinase ABC (Sigma) before incubation with proteins. One unit of each enzyme was added into each well and incubated for 2 h in PBS at 37°C under 5% CO₂. Then enzymes were removed by washing with PBS twice and incubated with fluos-labeled proteins. After the incubation, wells were washed three times with RPMI 1640 medium at 37°C to remove nonadherent proteins. Then cells were detached by using cell scrapers and collected in a tube and centrifuged at 300 × g for 5 min. After removing supernatant, cells were fixed by adding 1 ml of 1% paraformaldehyde-PBS. Fixed cells were analyzed by flow cytometry using CellQuest™ software (BD Biosciences).

**Construction of Mycobacteria Expressing Green Fluorescent Protein—**A BamHI-EcoRI 0.7-kb fragment from the vector pEGFP (BD Biosciences) containing the gene encoding GFP was introduced into the E. coli-mycobacteria shuttle vector pMV261 (23) to generate the plasmid pMV261-GFP. This plasmid was introduced into BCG by electroporation, and kanamycin-resistant BCG colonies were selected after 3 weeks of culture on Middlebrook 7H11 agar containing oleic acid, dextrose, albumin, and catalase enrichment (Difico, 7H11-OADC agar) in the presence of 20 μg/ml kanamycin. The expression of GFP was confirmed by confocal laser microscopy LSM510 (Carl Zeiss, Tokyo, Japan) according to the manufacturer’s instructions.

**Experimental Infection in Vitro—**Mycobacterial suspension was prepared by the conventional method (24) after harvesting bacteria in 7H9 media (Difico) supplemented with 10% albumin, dextrose, and catalase enrichment (Difico) and 0.05% Tween 80 at 37°C until 0.6 of optical density at 650 nm. Bacterial suspensions were added to A549 epithelial cells at a molar ratio of 10 to 1 to 10 bacteria/epithelial cell. After either 6- or 24-h incubation, unbound bacteria were washed with RPMI 1640 medium three times, and adherent epithelial cells were collected by scraping with rubber policeman. Serial 10-fold dilutions of the cell suspension were cultured on 7H11-OADC agar. Colonies were counted after 3 weeks incubation at 37°C. Cells infected with BCG expressing GFP were assessed by flow cytometry (FACScan, BD Biosciences) as described in the fluos-labeled protein binding assay.

**Experimental Infection in Vivo—**Female C57BL/6 mice, 7 weeks of age (Japan Clea, Suita, Osaka, Japan), were challenged intratracheally with 1 × 10⁶ CFUs of BCG in the presence or absence of 50 μg of HA. At 2 weeks of infection, lungs of mice were removed aseptically and homogenized individually with a set of motor and pestle. The homogenate was plated on 7H11-OADC agar after 10-fold serial dilution. Mycobacterial colonies were counted 3 weeks after the culture.

**Statistical Analyses—**Data were analyzed by Power Macintosh G4 using StatView 5.0 (SAS Institute Inc., Cary, NC) and expressed as the mean ± S.D. Data that appeared statistically significant were compared by an analysis of variance for comparing the means of multiple groups and considered significant if p values were less than 0.05.

**RESULTS**

**Cellular Localization of MDPI**—We examined cellular localization of MDPI by both biochemical and ultrastructural analyses. Subcellular fractions obtained from BCG were examined by Western blot analysis using anti-MDPI antisera (Fig. 1A). The result showed anti-MDPI antibody reacted with the bands in the cell wall and the membrane-ribosome fractions. In contrast, antibody failed to react with cytoplasmic fraction and culture filtrates. Next immunogold electron microscopic examination was carried out. Protein A-coupled with gold particles did not react with the section (Fig. 1B). In contrast, anti-MDPI antibody reacted with both inside and outside mycobacteria (Fig. 1C). These results indicate that MDPI localizes on/in the cell wall as well as intracellular ribosome and membrane fractions. This agrees with previous findings (11, 15).

**Amino Acids Sequence Homology between MDPI and HBHA—**Sequence alignments among BCG-MDPI, M. tuberculosis-MDPI, and M. tubercolis-HBHA were carried out by a maj ignorant program through the DNA data base of Japan (Fig. 2). BCG-MDPI revealed a lack of nine amino acids from M. tuberculosis-MDPI, and the identity of amino acid sequences was 95%. The total sequence homology between MDPI and HBHA was low (BCG-MDPI and HBHA, 35%; M. tuberculosis-MDPI and HBHA, 34%), although both proteins possessed the conserved region in the C-terminal region (Fig. 2). HBHA possessed a heparin-binding site in the C-terminal region, and four PAKK repetitive sequences are key residues for binding to GAGs (25,
Six PAKK repetitive sequences were seen in the C-terminal region of MDP1 (Fig. 2).

**MDP1 Binds to Heparin**—To explore the role of extracellularly occurring MDP1, we examined the binding capacity of MDP1 to heparin. Acid-soluble proteins enriched MDP1 derived from BCG (11) were applied on heparin-Sepharose chromatography, and heparin-binding proteins were fractionated by chromatography with a linear gradient from 0.15 to 2 M NaCl (Fig. 3A). Major 28-kDa protein on SDS-PAGE analysis was eluted by the fraction peak at 1.1 that was equivalent to 1 M NaCl. This protein was confirmed as MDP1 by Western blotting analysis utilizing monoclonal antibody (data not shown) and by determining the N-terminal amino acid sequences. It was reported that HABA was eluted at around 0.3 M NaCl by heparin-Sepharose column chromatography (26). Thus MDP1 is likely to bind to heparin with stronger affinity comparing to HBHA.

**SPR Analysis of the Interaction between MDP1 and GAGs**—To clarify the molecular interaction between MDP1 and GAGs, SPR analysis was performed. In the preliminary experiment, we found that MDP1 bound to both basic sensor chip C1 and dextran-coated sensor chip CM5. Based on this result, we subjected MDP1 as an immobilized ligand in the subsequent experiments.

Native MDP1 and antigen 85B, a 28-kDa fibronectin-binding protein (27, 28), were immobilized on the CM5 sensor chip until gaining 3332 and 3312 resonance units (RUs), respectively. Heparin (10 μg/ml), as an analyte, was injected into the control sensor cell and immobilized cells with either MDP1 or antigen 85B for 20 s. As expected from the result of heparin-Sepharose column chromatography analysis, the interaction between MDP1 and heparin was observed until gaining 300 RU. Heparin did not interact with antigen 85B and empty sensor.

To know whether MDP1 binds to possible receptors for mycobacteria in adherence to epithelial cells, we extended the study to other GAGs. It was previously reported that, in addition to heparin, adherence to Chinese hamster ovary (CHO) cells was inhibited by chondroitin sulfate, but not dextran, mannos, and galactose (6). Accordingly we examined these carbohydrates by SPR analysis. The results showed that chondroitin sulfate A and chondroitin sulfate C bound to MDP1. The maximum RUs were 63 and 55, respectively. In contrast, other carbohydrates such as dextrose, mannos, galactose, arabinose, and trehalose did not bind to MDP1 (data not shown). These data show that MDP1 binds to certain GAGs that are possible receptors for adherence to CHO cells (6). Also, MDP1 bound to HA and heparan sulfate with 90 and 89 RU, respectively (data not shown). All carbohydrates tested failed to bind to empty sensor or antigen 85B immobilized sensor.

Next we attempted to determine the affinity between MDP1 and GAGs. Because of the limitation of employing GAGs of certain molecular size, affinities were determined for heparin (M, 5,000, Fig. 3B) and heparan sulfate (M, 10,000, Fig. 3C) (Table I). Because true binding affinity cannot be determined for the ligand with multivalent, pseudo apparent rate constants (kₐ and k₈) were calculated by the basic model. The affinity (k₇) of heparin and heparan sulfate was 3.53 × 10⁻¹⁰ and 1.54 × 8⁻¹⁰, respectively.

Previously, real-time association between HBHA and heparin was studied by SPR analysis (26). In comparison to that, MDP1 associates with heparin more quickly and dissociates more slowly (HBHA to heparin, k₈; 5.62 × 10⁻⁵ s⁻¹, k₈; MDP1 to heparin, k₈; 5.62 × 10⁻⁷ s⁻¹, k₈; 5.62 × 10⁻⁹ s⁻¹).

**Bar**

**Fig. 1.** Cellular localization of MDP1 in mycobacteria. A, subcellular localization of MDP1 analyzed by the Western blot. Subcellular fractions were reacted with anti-MDP1 antisera on the transferred membrane after fractionated by SDS-PAGE. Lanes: 1, cell lysate of BCG; 2, cell wall fraction; 3, membrane-ribosome fraction; 4, cytoplasmic fraction; 5, culture filtrate. B and C, immunogold electron microscopic examination. B, control, pre-immune rabbit sera reaction. C, anti-MDP1 antiserum reaction. Magnification factor is ×20,000. Bar, 1 μm.

**Fig. 2.** MDP1 and HBHA possess PAKK repetitive sequences in the C-terminal regions. The alignment of amino acid sequences allowing gaps (−) between MDP1 (BCG-MDP and M. tuberculosis-MDP1) and HBHA. The alignment was performed by malgin program through DDBJ DNA data base. Asterisks indicate conserved among three proteins. Blue and underlined sequences represent the MD1-specific DNA binding region previously determined (8). Repetitive aa sequences, PAKK, are highlighted by red. Mtb. M. tuberculosis.
Determination of the Heparin-binding Site of MDP1—The difference of affinity between MDP1 and HBHA may be explained by binding sites other than PAKK repetitive regions in MDP1. To determine the binding site, we examined the heparin-binding activity of 20-mer of synthetic peptides covering the entire sequence of MDP1 (12). Wells of microtiter plates were coated with synthetic peptides and reacted with biotin-labeled heparin. Heparin bound to the peptide corresponding to an amino acid sequence of MDP1 at the 31–50 position (P31–50) (Fig. 4).

Exogenous DNA Inhibits MDP1-GAG Interaction—To provide direct evidence that MDP1 shares DNA- and GAG-binding sites, an inhibition assay was carried out by ELISA. As expected from SPR analysis, binding of MDP1 to GAG was detected as shown in Fig. 5. Binding of MDP1 to GAGs was inhibited by exogenous DNA, similar to the inhibition of MDP1-DNA interaction by DNA (Fig. 5). These results have confirmed the binding activity of MDP1 to GAGs and also showed that MDP1 interacts with GAGs by its DNA-binding site. The inhibition assay was carried out by ELISA. As expected from SPR analysis, binding of MDP1 to GAG was detected as shown in Fig. 5.
A549 cells with these enzymes resulted in a reduction of the binding (untreated, 93.0%; treated with heparinase 1, 38.5%; chondroitinase ABC, 26.8%; hyaluronidase, 5.8%; and the combination of enzymes, 3.7%) (Fig. 7). These results indicate that MDP1 binds to HA on A549 cells.

Inhibition of MDP1-A549 Cell Interaction by Exogenous DNA—We have already demonstrated that the DNA-binding region of MDP1 interacts with heparin (Figs. 4 and 5). The result prompted us to examine the possibility that the region may participate in the binding to A549 epithelial cells. The addition of exogenous DNA inhibited the binding of MDP1 (Fig. 8), but not HBHA (data not shown), to A549 cells. Based on the fact that MDP1 and its fragment, P31–50, bound to DNA via guanine and cytosine (11), we examined the effect of oligonucleotide DNA with 20-mer of dideoxyguanine (poly(dG)) on the interaction between MDP1 and A549 cells. The binding was inhibited by the addition of oligonucleotide DNA, although the intensity was less than plasmid DNA (Fig. 8). Taken together, these results suggest that MDP1 can bind A549 epithelial cells with its DNA-binding site.

Involvement of MDP1 and GAG Interaction in the Attachment/Invasion of Mycobacteria to A549 Cells—To elucidate the role of MDP1 in the attachment/invasion of mycobacteria, A549 epithelial cells were infected with BCG expressing GFP in vitro. CFUs determination showed around 7% of inoculated bacteria bound to cells after 6-h incubation. The interaction was then visualized by confocal laser microscopy. The result showed around 60% of bacteria bound to cells and 40% was invaded. Thus BCG not only bound to but also invaded into cells. This was consistent with previous reports (4, 32). In this condition, exogenous HA, heparin, DNA, and anti-MDP1 antibodies inhibited the interaction between GFP-expressing BCG and A549 cell (Fig. 9A). P31–50 suppressed BCG-A549 cell interaction as well (data not shown). Among these, the most potent inhibitor of the interaction was HA. Similar results were obtained from the experiment involving M. tuberculosis (Fig. 9B).

Inhibition of BCG Growth in Vivo by Treatment of Mice with HA—We have extended in vitro studies to in vivo animal experiments regarding the inhibitory role of GAGs in the interaction. Mice were injected intratracheally with either 1 × 10⁶ CFU of BCG alone or BCG plus 50 μg of HA. Two weeks after the challenge, 40,875 ± 16,585 CFU was recovered from the lungs of BCG-infected mice (Fig. 10). Treatment of such mice with HA resulted in a marked reduction of BCG growth (5,960 ± 4,530 CFU). This result was consistent with that of in vitro study, probably due to the prevention of mycobacterial attachment/invasion by exogenous HA.

**DISCUSSION**

The bacterial chromosomes are associated with abundant histone-like proteins that compactly hold the genome (33–35). It is generally accepted that such histone-like proteins participate functionally in the regulation of gene expression by altering three-dimensional genome structure (33–35). MDP1 is a presumed mycobacteria-specific histone-like protein, although it localizes on the cell wall, besides inside the cell (11, 15). In the present study we have focused on the physiological role of extracellularly occurring MDP1. We postulated that surface-exposed MDP1 acts as an adhesin in Mycobacterium-epithelial cell interaction based on the following facts. First, GAG is estimated as an infectious site of mycobacteria to epithelial cells (6). Second, DNA-binding protein tends to bind GAGs, as for example the heparin-Sepharose column is conventionally used to purify nuclear proteins. Third, MDP1 possesses six PAKK sequences that represent the heparin-binding site of HBHA (Fig. 2).

In the present study we explored interaction between MDP1 and GAGs and found that MDP1 directly bound to GAG at the DNA-binding site (Figs. 3–5). Affinity was determined by SPR analysis. Apparent rate constants were calculated by the basic model, because heparin and heparan sulfate are multivalent. There is a discrepancy at least in part by difference between the fitted model and real interaction mechanism and requires further investigation to obtain the actual kinetic constants for the interaction. MDP1 bound to A549 lung epithelial cells mainly through cell surface HA (Fig. 7). Anti-MDP1 antibody treatment inhibited the binding of mycobacteria, including BCG and M. tuberculosis, to A549 cells (Fig. 9). These findings indicate that MDP1 acts as an adhesin in the interaction with lung epithelial cells of the host. To our knowledge, this study demonstrates for the first time that bacterial pathogen utilizes extracellular DNA-binding proteins to attach host cells.

It has also been known that HBHA binds to GAGs and is identified as a mycobacterial adhesin (6). MDP1 and HBHA are structurally distinct proteins, although both proteins possess the conserved repetitive PAKK sequences in the C terminus (Fig. 2). Functionally, MDP1 binding to A549 cells was seen immediately after its addition, and >95% of A549 cells were MDP1-positive 60 min thereafter. In contrast, binding of HBHA was delayed, and >10% of cells were HBHA-negative.
even at the plateau phase (Fig. 6B). The possible mechanism of the functional difference can be explained by the affinity experiment, which showed that MDP1 possessed stronger binding activity to GAGs than HBHA, as assessed by heparin Sepharose column chromatography and BIAcore biosensor analysis (Fig. 3) (26). Another possible mechanism is the distinct binding site of MDP1 and HBHA to GAGs. The binding site of MDP1 comprises aa 31–50, which is the DNA binding region, but not the region of six repetitive sequences of PAKK in its C terminus (Fig. 4), whereas that of HBHA comprises four repetitive sequences of PAKK (20, 21). As expected, exogenous DNA inhibited the binding of MDP1, but not HBHA, to A549 cells (Fig. 8 and data not shown). The interaction between MDP1 and A549 cells was through cell surface HA (Fig. 7), whereas heparan sulfate is presumed to be the site for HBHA (26).

Another major difference between MDP1 and HBHA is their localization. MDP1 retained mycobacterial cell walls, although HBHA is released to the extracellular milieu. The nature of MDP1 having strong affinity to both cell walls and GAG may be more favorable than HBHA to attach to host tissues. Recently Coutte et al. (36) found that the release of adhesin to the extracellular milieu is necessary for efficient colonization of Bordetella pertussis, which colonizes to the human respiratory

![Fig. 6. MDP1 binds to A549 human lung epithelial cells. A, phase contrast microscopy image (a) and corresponding fluorescent microscopic image (b), c, merge of a and b. B, binding kinetics of MDP1 and HBHA to A549 cells. Fluorescent-labeled MDP1 (0.5 μM) was incubated with A549 cells for 1 h. MDP1- and HBHA-bound cells were determined by FACSscan at each time point after the addition of proteins (horizontal axis). C, heparin and HA, but not mannose, inhibited the binding of MDP1 to A549 cells. MDP1 was pre-incubated with HA, heparin, and mannose and was then added into the culture of A549 cells. After 2-h incubation, the % of MDP1-bound cells was determined by FACSscan. D, inhibition by HA and heparin of the binding of MDP1 (left panel) and HBHA (right panel) to A549 cells.](http://www.jbc.org/)

![Fig. 7. MDP1 binds to A549 cells through HA. Untreated A549 cells bound to fluo-BSA served as negative controls (A) and to fluo-MDP1 as positive controls (B). MDP1 was added to the culture of cells pretreated with heparinase 1 (C), chondroitinase ABC (D), hyaluronidase (E), and combination of these three enzymes (F). Intensity of fluorescence was determined by FACSscan 4–5 h after the culture.](http://www.jbc.org/)
Mycobacterium-Lung Epithelium Interaction

HBHA-lacking BCG and mice with HA.

C57BL/6 mice were challenged intratracheally with A/H11003 either 1 duplicated samples was presented. The binding was assessed by FACScan. The average of cells. As controls for antibody experiments, rabbit control Ig (Sigma) bodies, HA, and heparin, and were then added into the culture of A549 GFP to A549 cells. BCG were pretreated with DNA, anti-MDP1 anti-

uptake with A549 cells. Bacterial numbers were counted 3 weeks after the treated with GAGS and anti-MDP1 antibodies recovered from the coculture with A549 cells. Bacterial numbers were counted 3 weeks after the culture on the Middlebrook 7H11 agars. Mtb, M. tuberculosis. *, p < 0.05 and **, p < 0.01 as compared with Mtb alone by analysis of variance.

Fig. 9. MDP1 is involved in the attachment/binding of mycobacteria to A549 cells. A, the attachment/binding of BCG expressing GFP to A549 cells. BCG were pretreated with DNA, anti-MDP1 antibodies, HA, and heparin, and were then added into the culture of A549 cells. As controls for antibody experiments, rabbit control Ig (Sigma) was added. The binding was assessed by FACScan. The average of duplicated samples was presented. B, CFU of M. tuberculosis pretreated with GAGs and anti-MDP1 antibodies recovered from the coculture with A549 cells. Bacterial numbers were counted 3 weeks after the culture on the Middlebrook 7H11 agars. Mtb, M. tuberculosis. *, p < 0.05 and **, p < 0.01 as compared with Mtb alone by analysis of variance.

Fig. 10. Inhibition of infection of BCG in vivo by treatment of mice with HA. C57Bl/6 mice were challenged intratracheally with either 1 × 106 CFU BCG alone (C) or BCG plus 50 μg of HA (+HA). All results are expressed as individual mouse data. Bars indicate averages. p = 0.0143 as compared between two groups by Mann-Whitney U test.

tract. HBHA induces bacterial aggregation, thereby mediating bacteria-bacteria interaction (6). These reports suggest that exported HBHA participates in the dispersal of bacteria from microcolonies for the spread of infection. This hypothesis explains the important finding by Pethe et al. (8): delay of dissemination of HBHA-lacking BCG and M. tuberculosis from the lung to spleen. Taking these considerations together, mycobacterium possess at least structurally and functionally two distinct adhesion proteins, such as extracellular MDP1 and HBHA.

It remains uncertain how MDP1 is expressed on cell walls of mycobacteria. An MDP1 gene does not encode signal sequence of secretion, and it does not contain the transmembrane domain of integration into the cell membrane. However, the large amounts of MDP1 that were localized on/in mycobacterial cell walls (Fig. 1) imply that some machinery is present that transports MDP1 outside the cell membrane rather than retention on the cell surface due to cell lysis of bacteria. Certain proteins that lack signal sequence and a transmembrane domain, such as ESAT-6 (37), CFP-10 (38), HSP65 and superoxide dismutase (SOD) (39, 40), and glutamine synthetase (39), are actively exported from mycobacteria and play significant roles in the pathogenesis. Further study is needed to clarify the issue of transportation of MDP1.

Hyaluronidase treatment of A549 cells abolished MDP1-A549 cell interaction (Fig. 7), and exogenous addition of HA reduced the binding of BCG and M. tuberculosis to A549 cells (Fig. 9), suggesting that cell surface HA is the key GAG for adherence of mycobacteria to lung epithelial cells. Mycobacterial adhesins other than MDP1 are likely to participate in binding to HA, because anti-MDP1 antibodies and DNA, inhibited the interaction between mycobacteria and A549 cells less than HA (Fig. 9A).

Based on the results of in vitro experiments, we have attempted the therapeutic intervention by HA using experimental infection with BCG in mice. Treatment of such mice with HA resulted in a marked reduced BCG growth in the lung (Fig. 10). The results support the in vitro study that HA plays an important role in the interaction between mycobacteria and lung epithelial cells. This suggests that HA has potential for prophylactic interventions in mycobacterial infection.

HA has so far received little attention in the research of host-Mycobacterium interaction. HA is a polymer comprising repeating disaccharide units of (β1–4)-N-glucuronate (β1–3)N-acetyl-D-glucosamine (7). HA is the major component of the extracellular matrix and acts as a signaling molecule for cells depending on their size (41, 42). Professional phagocytes, such as macrophages, and professional antigen presenting cells, such as dendritic cells, play important roles in defense against mycobacterial infection in the lung (43). HA modulates the functions of dendritic cells and macrophages (42). Fragmented HA, which accumulates during inflammation, stimulates nitric oxide production (44), which is the major bactericidal effector against M. tuberculosis (43, 45). Furthermore, CD44, a major receptor of HA (46, 47), is recently identified as the site of mycobacterial entry to macrophages (48). We focused mainly on mycobacteria-epithelial cell interaction in this study, and our findings imply that HA plays an important role in interactions between mycobacteria and dendritic cells. The precise mechanism of the interaction remains to be elucidated, and such study is currently underway in our laboratory.

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