Characterization of the Heparin-binding Site of the Mycobacterial Heparin-binding Hemagglutinin Adhesin*  

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The mycobacterial adhesin heparin-binding hemagglutinin (HBHA) contains several lysine-rich repeats at its carboxyl-terminal end. Using truncated recombinant HBHA forms and hybrid proteins containing HBHA repeats grafted onto the Escherichia coli maltose-binding protein (MBP), we found that these repeats are responsible for heparin binding. Immunofluorescence microscopy studies revealed that their deletion abrogates binding of HBHA to human pneumocytes. Conversely, when fused to MBP, the HBHA repeats confer pneumocyte adherence properties to the hybrid protein. Treatment of pneumocytes with glycosaminoglycan-degrading enzymes showed that HBHA binding depends on the presence of heparan sulfate chains on the cell surface. The epitope of a monoclonal antibody that inhibits mycobacterial adherence to epithelial cells was mapped within the lysine-rich repeats, confirming their involvement in mycobacterial adherence to epithelial cells. Surface plasmon resonance analyses showed that recombinant HBHA binds to immobilized heparin with fast association kinetics ($k_a = 5.62 (\pm 0.10) \times 10^5 \text{ M}^{-1} \text{s}^{-1}$), whereas the dissociation kinetics were slower ($k_d = 0.015 (\pm 0.002) \text{s}^{-1}$), yielding a $K_D$ value of 26 nM. Similar analyses with grafted MBP indicated similar kinetic constants, indicating that the carboxyl-terminal repeats contain the entire heparin-binding site of HBHA. The molecular characterization of the interactions of HBHA with epithelial glycosaminoglycans should help to better understand mycobacterial adherence within the lungs and may ultimately lead to new approaches for therapy or immunoprophylaxis.

Mycobacteria are among the most successful pathogenic microorganisms for humans. Mycobacterium tuberculosis infects over one-third of the population in the world, causing annually over 10 million new cases of active tuberculosis and 3 million deaths (1, 2). Leprosy, caused by Mycobacterium leprae, remains a major health problem in developing countries (3), whereas members of the Mycobacterium avium-intracellulare complex are among the most frequent opportunistic pathogens infecting patients suffering from acquired immunodeficiency syndrome (4).

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‡‡‡‡ Despite the importance of mycobacterial diseases, their molecular mechanisms are still poorly understood. One of the initial and crucial events in any infectious process is the adherence of the microorganism to its target tissues (5). Mycobacteria exhibit a tropism for the lungs, and interactions of the tubercle bacillus with alveolar macrophages have been extensively documented (6–8). However, mycobacterial adhesins interacting with respiratory epithelial cells or extracellular matrix may also play a role in the infection, because these are the first host tissues encountered by mycobacteria when they are transmitted by aerosol. As described for other pathogenic bacteria (9, 10), viruses (11, 12), and parasites (13, 14), M. tuberculosis produces on its surface a heparin-binding protein involved in adherence to epithelial cells (15). Because of its capability to agglutinate rabbit erythrocytes, this adhesin was called heparin-binding hemagglutinin (HBHA).1 HBHA is a 199-residue glycoprotein that also induces bacterial autoaggregation (15–17). Recombinant HBHA (rHBHA) produced by Escherichia coli is not glycosylated and, in contrast to the native protein, is highly sensitive to proteolytic degradation affecting essentially its carboxyl-terminal end. This part of the protein contains two different lysine-rich repeated motifs (16), named R1 and R2. R1 (KKAAAP) is directly repeated thrice between residues 160 and 177, whereas R2 (KKAAAAKK) is repeated twice between amino acids 178 and 194. In this report, we show that these repeats constitute the high affinity heparin-binding site of HBHA and that they are responsible for the binding of HBHA to heparan sulfate glycosaminoglycans (GAGs) present on the surface of human pneumocytes.

Experimental Procedures

Bacterial Strains, Growth Conditions, and DNA Manipulations—Growth conditions for Mycobacterium bovis BCG (strain 1173P2; World Health Organization, Stockholm, Sweden) have been previously described (5). E. coli XL1-Blue (New England Biolabs, Beverly, MA), E. coli BL21(DE3) (Novagen, Madison, WI), and E. coli B834 (18) were grown in LB medium (19) supplemented with 100 μg/ml ampicillin or 30 μg/ml kanamycin when appropriate. Restriction enzymes, T4 DNA ligase, and other molecular biology reagents were purchased from New England Biolabs, Roche Molecular Biochemicals, or Promega (Madison, WI). Polymerase chain reactions (PCRs) were performed using a Perkin-Elmer thermal cycler model 480 (Perkin-Elmer) with 100 ng of primer (Table I). All the PCR fragments were sequenced using an ABI PRISM Dye Terminator Cycle Sequencing kit and an ABI PRISM 377 sequencer (Perkin-Elmer). Plasmids were purified on Nucleosbond AX 100 cartridges (Macherey-
To purify rHBHA/C, cells from a 250-ml culture of E. coli BL21(DE3)pLysET-HBHA/C were resuspended into 50 ml of 50 mM Tris-HCl (pH 8.0) and sonicated twice for 5 min at 4 °C. The soluble fraction obtained after centrifugation (7000 × g for 15 min at 4 °C) was loaded at a flow rate of 0.5 ml/min onto a DEAE-cellulose (DE 52, Whatman, Maidstone, Kingdom) column (1 × 4 cm). The column was equilibrated with 150 ml of 50 mM Tris-HCl (pH 8.0). HBHA/C was eluted using a linear 0–1 mM NaCl gradient in 100 ml of 50 mM Tris-HCl (pH 8.0). The material eluted between 150 and 200 mM NaCl was collected, diluted twice with 50 mM Tris-HCl (pH 8.0), 4 mM NaCl, and finally loaded at a flow rate of 0.5 ml/min onto a Phenyl Sepharose CL-4B (Amersham Pharmacia Biotech) column (1 × 5 cm) equilibrated with 100 ml of 50 mM Tris-HCl (pH 8.0), 2 mM NaCl. rHBHA/C was eluted with a negative linear 2–0 mM NaCl gradient in 100 ml of 50 mM Tris-HCl (pH 8.0). The fractions eluted between 0.8 and 0.6 mM NaCl contained purified rHBHA/C, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) performed according to Laemmli (20) by using a 4% stacking and 12 or 15% separating gels. After electrophoresis, gels were stained with Coomassie Brilliant Blue R-250 (Merck). Protein concentrations were determined according to the method of Bradford (21), using bovine serum albumin (BSA) as a standard.

**Table I**

| HBHA (5') | 5'-ATCCTCTGGGGACTCGACG-3' |
| HBHA (3') | 5'-TATAGACCGTACCGTAAGACGACGTTTGC-3' |
| HBHA + 1 R (SacI) | 5'-TATAGACGCTAGTGGAGCAAGACGACGTTTGC-3' |
| HBHA + 2/3 R (SacI) | 5'-TATAGACGCTAGTGGAGCAAGACGACGTTACGACG-3' |
| E4 (5') | 5'-ATATGTCAGAAGAGGTGCTGTGGGCACTCGACCG-3' |
| E4 (3') | 5'-CTAGGACTGCAACTGCCATGCTGGG-3' |

Nagel, Düren, Germany) according to the instructions of the manufacturer.

**Plasmid Construction**—To produce rHBHA under the control of the T7 promoter, the 643-base pair NcoI-HindIII fragment of pKK-HBHA (16) was introduced into NcoI/HindIII-restricted pET24d(+) (Novagen) and the resulting plasmid, pET-HBHA, was used to transform E. coli BL21(DE3). The gene coding for a truncated HBHA (rHBHA/C) lacking amino acids 161–199 was generated by PCR using pKK-HBHA as template and the oligonucleotides HBHA (5') and HBHA/C as primers (Table I). The 492-base pair PCR fragment was digested with NcoI and SacI and ligated into NcoI/SacI-restricted pET24d(+) (CLONTECH, Palo Alto, CA) and pET24d(+) to generate pKK-pET-HBHA/C and pET-HBHA/C, respectively. pKK-HBHA/C was used to transform E. coli XLI-Blue, and pET-HBHA/C was introduced into E. coli BL21(DE3).

Genes coding for HBHA with one (rHBHA/C+1R1), two (rHBHA/C+2R1), or three (rHBHA/C+3R1) R1 motifs were generated by PCR using pKK-HBHA as template and oligonucleotides HBHA (5') with HBHA/C+1R1 or HBHA/C+2R1 or R1 primers. After purification and restriction by NcoI and SacI, the PCR fragments were inserted into NcoI/SacI-restricted pET24d(+), generating pET-HBHA/C+1R1, pET-HBHA/C+2R1, and pET-HBHA/C+3R1, respectively. These plasmids were used to transform E. coli BL21(DE3). Genes encoding maltose-binding protein (MBP) containing various combinations of HBHA repeats (MBP+3R1+2R2 and MBP+1R1+2R2) were generated as follows. First, the 3'-region of the HBHA gene coding for the repeats was amplified by PCR using pKK-HBHA, and oligonucleotides E4 (5') and E4 (3'). Oligonucleotide E4 (5') has three hybridization sites on the template DNA. However, the PCR generated only two products of 222 base pairs and 186 base pairs that correspond to amino acids 161–199 (3R1+2R2) and amino acids 173–199 (1R1+2R2), respectively. After digestion with EcoRI and PstI, the PCR fragments were ligated into EcoRI/PstI-digested pMAL (New England Biolabs) to generate pMAL(+)pET-HBHA/C+1R1, pMAL(+)pET-HBHA/C+2R1, and pMAL(+)pET-HBHA/C+3R1, respectively. These plasmids were then used to transform E. coli B834 and E. coli XLI-Blue.

**Production of Recombinant Proteins in E. coli**—Recombinant HBHA with or without the carboxyl-terminal truncations, as well as MBP, MBP+3R1+2R2, and MBP+1R1+2R2 (Table II) were produced in E. coli. After transformation with the appropriate recombinant plasmid, E. coli cells were grown at 37 °C in 250 ml of LB broth supplemented with ampicillin or kanamycin. At an A600 of 0.5, isopropylthiogalactoside was added to a final concentration of 1 mM, and incubations were continued for 4 h. The cultures were then centrifuged for 15 min at 4 °C and 7000 × g. The supernatants were discarded, and the cells were resuspended into 20 ml of phosphate-buffered saline (PBS; 140 mM NaCl, 5 mM KCl, 20 mM Na2HPO4, 3.5 mM KH2PO4). Cell suspensions were stored at −20 °C until further use.

**Protein Purification**—To purify the rHBHA, frozen recombinant E. coli cell suspension was thawed and sonicated twice for 5 min at 4 °C using a Branson Sonifier at an output of 5 delivered to a microtip. The bacterial lysates were then centrifuged at 10,000 × g for 15 min at 4 °C. The supernatants were diluted to 100 ml with PBS and then applied onto a heparin-Sepharose CL-6B (Amersham Pharmacia Biotech) column (1 × 4 cm) which was then equilibrated with 100 ml of PBS. The bound material was eluted with a 0–500 mM NaCl linear gradient prepared in 80 ml of PBS. The column was regenerated by washing the gel with 30 ml of PBS containing 3 mM NaCl. All chromatographic steps were carried out at room temperature using a flow rate of 1.5 ml/min. Native M. bovis BCG HBHA was purified by heparin-Sepharose chromatography as described previously (15).
bated for 20 min at room temperature in 1 ml of fluorescein isothiocyanate-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) diluted 500-fold. After 3 final washes with PBS/BSA, the cells were fixed with 500 μl of 4% paraformaldehyde, and cell-associated fluorescence (percentage of fluorescein isothiocyanate-labeled pneumocytes) was quantified by flow cytometry using an EPICS Elite cytometer (Coulter, Hialeah, FL). Fluorescence associated with pneumocytes that were not incubated with rHBHA was used to determine nonspecific signals.

**Immunoblot Analyses**—After SDS-PAGE, proteins were transferred onto nitrocellulose membranes (BA 85; Schleicher & Schuell) as described by Towbin et al. (22). Immobilized native HBHA, rHBHA, and truncated versions of rHBHA, MBP, or MBP-HBHA hybrid proteins were then probed with purified anti-HBHA monoclonal antibody (mAb) 4057/D2 or 3921E4 (23), 500-fold diluted anti-HBHA rat serum (15), or 10,000-fold diluted rabbit anti-MBP serum (New England Biolabs). mAb 4057/D2 was purified by cryoprecipitation (24), and mAb 3921E4 was purified by protein A-Sepharose Fast Flow (Amersham Pharmacia Biotech) chromatography. Immune complexes were developed with alkaline phosphatase-linked goat anti-mouse, anti-rat, or anti-rabbit IgG (Promega, Madison, WI). To investigate the influence of dextran (500 kDa, Sigma), dextran sulfate (500 kDa, Sigma), and heparin (5 kDa, Sigma) on the immunoreactivities, nitrocellulose membranes bearing immobilized antigens were incubated at room temperature for 1 h in PBS containing 500 μg/ml of the polysaccharide to be tested. After two washes with PBS, the membranes were probed with mAb 3921E4 or anti-MBP serum and developed as described above.

**RESULTS**

**Dextran Sulfate Protects rHBHA from Proteolytic Degradation**—Unglycosylated rHBHA produced in *E. coli* undergoes rapid proteolytic degradation affecting its carboxyl-terminal lysine-rich repeats (16). This degradation correlates with a reduction of affinity for heparin, raising the possibility that these repeats are involved in heparin binding by HBHA. To test this hypothesis, dextran sulfate (500 kDa) or dextran (500 kDa) was added at 500 μg/ml to an *E. coli* XL1-Blue (pKK-HBHA) lysate. As shown in Fig. 1, dextran sulfate but not dextran partially protected rHBHA from proteolysis during incubation at 37 °C. A decrease in dextran sulfate concentration correlated with a reduction of the protective effect (data not shown). These observations suggest that the sulfated polysaccharide masked proteolytic cleavage sites through its binding to the carboxyl terminus of rHBHA. Low molecular mass heparin (3 kDa) did not protect rHBHA, even at concentrations up to 2 mg/ml, indicating that the length of the sulfated polysaccharide may be important to protect rHBHA from proteolytic degradation.

**High Level Production of rHBHA by a Protease-deficient *E. coli* Strain**—To circumvent the degradation of rHBHA, the protein was produced under the control of the T7 promoter in *E. coli* BL21(DE3), a protease-deficient strain. No significant rHBHA degradation was observed during the first 4 h in an *E. coli* BL21(DE3)(pET-HBHA) lysate incubated at 37 °C, whereas under the same conditions all rHBHA expressed in *E. coli* XL1-Blue was cleaved into a 19-kDa polypeptide (16). After 24 h of incubation, less than 50% of the rHBHA was cleaved into a 25-kDa polypeptide in the *E. coli* BL21(DE3)(pET-HBHA) lysate (data not shown). These results show that a protease-deficient *E. coli* strain allows for stable production of rHBHA. Moreover, the use of the T7 promoter instead of the trc promoter permitted a ~10-fold increase in rHBHA production, as determined by densitometric analysis of the Coomassie-stained electrophoresis gels.

**rHBHAΔC Does Not Bind to Heparin and Is Not Recognized by mAb 3921E4**—The production of rHBHAΔC in *E. coli* XL1-Blue (pKK-HBHAΔC) and in *E. coli* BL21(DE3)(pET-HBHAΔC) was analyzed by SDS-PAGE and immunoblotting. Similar to full-length rHBHA, the production of rHBHAΔC was strongly enhanced when the recombinant gene was under the control of the T7 promoter (Fig. 2, compare lanes E and G). In both expression systems, the apparent molecular mass of rHBHAΔC was ~25.4 kDa, whereas its calculated molecular mass is 17.4 kDa. This difference is similar to that observed for full-length rHBHA (16), indicating that the deletion of the repeated motifs does not abolish the aberrant electrophoretic migration. Incubation of *E. coli* XL1-Blue (pKK-HBHAΔC) lysates for 1 day at 37 °C revealed that rHBHAΔC does not undergo degradation (data not shown), consistent with the notion that the proteolytic susceptibility of full-length rHBHA concerns the lysine-rich repeats. mAb 3921E4, which blocks the heparin-inhibitable binding of *M. tuberculosis* to epithelial cells (15, 16), failed to recognize rHBHAΔC (Fig. 2, lanes E), indicating that at least part of its epitope is located within the HBHA repeats. To determine whether these repeats are involved in the interaction of HBHA with heparin, an *E. coli* BL21(DE3)(pET-HBHAΔC) lysate was applied onto a heparin-Sepharose column. All the rHBHAΔC was recovered in the flow-through fraction, whereas under the same conditions, full-length rHBHA produced by *E. coli* BL21(DE3)(pET-HBHA) remained bound to the column. These observations indicate that the lysine-rich motifs of HBHA are involved in the interaction with heparin.

**Deletions within the rHBHA Repeats Weaken the Interaction with Heparin**—To assess the contribution of the R1 repeats to the HBHA-heparin-binding activity and to further map the epitope of mAb 3921E4, rHBHAΔC+1R1, rHBHAΔC+2R1, and rHBHAΔC+3R1 were produced in *E. coli* BL21(DE3). Recombinant protein production was analyzed by SDS-PAGE and immunoblotting using mAb 3921E4 (Fig. 3). This antibody failed to recognize rHBHAΔC+1R1 and reacted only faintly with rHBHAΔC+2R1, whereas its reactivity with rHBHAΔC+3R1 was comparable to that observed with rHBHA, suggesting that the mAb 3921E4 epitope overlaps two successive R1 motifs. Interestingly, compared with rHBHA, the rHBHAΔC with 1–3 R1 motifs was less stable in *E. coli* BL21(DE3), suggesting that the missing carboxyl-terminal amino acids, perhaps the terminal VTQK sequence, may shield from proteolysis (Fig. 3, lanes C–E).

When tested for their ability to interact with heparin, rHBHAΔC+1R1 was found capable to bind to heparin-Sepharose, and rHBHAΔC+2R1 bound weakly because it could be eluted with 20 mM NaCl. The presence of the third R1 motif strengthened the interaction with heparin because elution of rHBHAΔC+3R1 required 100 mM NaCl. These results indicate that the R1 motifs are involved in heparin binding, but they are not sufficient for the high affinity binding observed for full-length rHBHA, suggesting that the missing 21 residues, per-
haps the R2 motifs, also play a role in the interaction with heparin.

Grafting of the HBHA Repeats onto MBP Confers Heparin Binding and mAb 3921E4 Recognition to the Hybrid Protein—To investigate whether the carboxyl-terminal repeats are sufficient for heparin binding, amino acids 161–199 (3R1 2R2) or 173–199 (1R1 2R2) of HBHA were fused to the carboxyl end of MBP. Production of the hybrid proteins MBP 1 3R1 2R2 and MBP 1 2R2 was confirmed by SDS-PAGE and Coomassie Blue staining.

**FIG. 1.** Inhibition by dextran sulfate of proteolysis of rHBHA produced in E. coli XL1-Blue. Cell extracts obtained by sonication of a 250-ml culture of E. coli XL1-Blue(pKK-HBHA) were incubated at 37 °C with dextran (500 kDa, left) or dextran sulfate (500 kDa, right) at a final concentration of 500 μg/ml. After 1 min (lanes A), 15 min (lanes B), 30 min (lanes C), 1 h (lanes D), and 3 h of incubation (lanes E), the proteins were analyzed by SDS-PAGE and Coomassie Blue staining. The position of rHBHA is shown by a star, and molecular mass markers expressed in kDa are indicated in the left margin.

**FIG. 2.** SDS-PAGE and immunoblot analyses of rHBHA and rHBHAΔC produced in E. coli XL1-Blue and E. coli BL21(DE3). E. coli XL1-Blue(pKK-HBHA) (lanes B and C), E. coli XL1-Blue(pKK-HBHAΔC) (lanes D and E), and E. coli BL21(DE3)(pET-HBHAΔC) (lanes F and G) lysates were analyzed before (lanes B, D, and F) and after induction by isopropylthiogalactoside (lanes C, E, and G). Lanes A contain 200 ng of purified HBHA from M. bovis BCG. After electrophoresis, the proteins were stained with Coomassie Blue R-250 (left and right panels) or transferred onto nitrocellulose membranes and probed with mAb 3921E4 (middle panel). The arrowheads indicate the positions of rHBHA (lane C) and rHBHAΔC (lane E). Molecular mass markers expressed in kDa are given in the left margin.

**FIG. 3.** SDS-PAGE and immunoblot analyses of rHBHA containing truncations within the carboxyl-terminal repeats. E. coli BL21(DE3)(pET24d(+) (lanes A), E. coli BL21(DE3)(pET-HBHA) (lanes B), E. coli BL21(DE3)(pET-HBHAΔC+3R1) (lanes C), E. coli BL21(DE3)(pET-HBHAΔC+2R1) (lanes D), E. coli BL21(DE3)(pET-HBHAΔC+1R1) (lanes E), and E. coli BL21(DE3)(pET-HBHAΔC) (lanes F) lysates were analyzed by SDS-PAGE and Coomassie Blue staining (left panel) and immunoblotting using mAb 3921E4 (right panel). Molecular mass markers expressed in kDa are shown in the left margin.
MBP+1R1+2R2 was analyzed by SDS-PAGE and immunoblotting. Both proteins were equally well recognized by mAb 3921E4, confirming that its epitope is located within the repeats. When produced in E. coli XL1-Blue, MBP+3R1+2R2 and MBP+1R1+2R2 were rapidly degraded into polypeptides of 41.5 and 40 kDa, respectively, which were resistant to further degradation and no longer recognized by mAb 3921E4 (data not shown).

The capacity of the MBP hybrid proteins to bind to sulfated polysaccharides was investigated by immunoblotting and heparin-Sepharose chromatography. As shown in Fig. 4, pretreatment of nitrocellulose membranes bearing MBP+3R1+2R2 with dextran sulfate but not with unsulfated dextran impeded the subsequent recognition by mAb 3921E4 but not that of anti-MBP antibodies, suggesting that the sulfated polysaccharide bound to the hybrid protein and masked the 3921E4 epitope. The size of the sulfated polysaccharide appeared to be important as low molecular weight heparin did not inhibit 3921E4 reactivity (data not shown). To determine whether the fusion of the HBHA repeats to MBP induces its binding to heparin, E. coli B834(pMAL-c), E. coli B834(pMAL+3R1+2R2), and E. coli B834(pMAL+1R1+2R2) lysates were subjected to heparin-Sepharose chromatography. Whereas MBP was unable to bind to heparin, MBP+3R1+2R2 bound to the column and was eluted with 300 mM NaCl (Fig. 5), a condition similar to that required for the elution of rHBHA. MBP+1R1+2R2 also bound to heparin-Sepharose but less strongly because it was eluted with 100 mM NaCl. These results indicate that the molecular determinants required for the heparin-binding activity of HBHA are all located within the carboxyl-terminal repeats and that the heparin-binding activity of HBHA can be transferred to an heterologous protein by tagging with the HBHA repeats.

SPR Analysis of the HBHA-Heparin Interactions—SPR was used to more precisely analyze the interaction of rHBHA and MBP+3R1+2R2 with heparin. As shown in Fig. 6, rHBHAΔC and MBP were unable to interact with the heparin sensor chip, whereas rHBHA and MBP+3R1+2R2 displayed comparable interaction profiles. The affinity constants calculated using protein concentrations of 200 nM were 26 (±2) and 23 (±1) nM for rHBHA and MBP+3R1+2R2, respectively, confirming that the high affinity binding of HBHA to heparin is entirely mediated by its lysine-rich repeats. For both proteins, the interaction with heparin was characterized by fast association kinetics. However, the association rate constant for the interaction of MBP+3R1+2R2 with heparin (k_a = 6.65 (± 0.15) × 10^5 M⁻¹ s⁻¹) was slightly higher than that calculated for the rHBHA-heparin interaction (k_a = 5.62 (± 0.10) × 10^5 M⁻¹ s⁻¹), suggesting perhaps a slightly faster binding for the hybrid protein. No difference was observed for the dissociation rate constants between the two proteins.

Binding of HBHA to Human Pneumocytes Is Mediated by the Carboxyl-terminal Repeats—To test whether the HBHA repeats are involved in HBHA adherence to human pneumocytes, purified rHBHA and rHBHAΔC were incubated with A549 cells, and protein binding was monitored by immunofluorescence microscopy. As shown in Fig. 7, rHBHA bound to the cell surface, whereas rHBHAΔC did not, indicating that the repeats are required for interaction of HBHA with human pneumocytes. In this binding assay, MBP did not bind to A549 cells, whereas MBP+3R1+2R2 bound to the pneumocytes (Fig. 7), indicating that the repeats are sufficient for binding of HBHA to respiratory epithelial cells.

Pneumocyte Heparan Sulfate Chains Act as Receptors for HBHA—To investigate the involvement of GAGs in HBHA binding to human pneumocytes, A549 cells were treated with chondroitinase ABC or heparinase III, which cleaves chondroitin sulfate A, B, and C or heparan sulfate, respectively. Untreated cells as well as lyase-treated cells were then incubated with rHBHA, and the adhesion binding was monitored by flow cytometry. Whereas the chondroitinase ABC treatment did not significantly reduce the interaction of HBHA with pneumocytes, the heparinase III treatment led to a reduction of 68% of rHBHA binding (Fig. 8), indicating that proteoglycans containing heparan sulfate chains serve as receptors for HBHA on the surface of human pneumocytes.

**DISCUSSION**

To mediate adherence during the early steps of infection, pathogenic microorganisms commonly interact with cell surface receptors that are normally recognized by eukaryotic ligands to mediate signal transduction, as well as cell-cell and
cell-extracellular matrix interactions (25). These molecules are generally glycoproteins, and an increasing number of bacterial adhesins are now being shown to express specific lectin activity for the carbohydrate moieties of these receptors (26). Among those carbohydrates, heparan sulfate and related sulfated polysaccharides such as chondroitin sulfate are of special interest, because they are present on the surface of virtually all animal cells in the form of sulfated proteoglycans (27). Indeed, the GAG chains of proteoglycans constitute a family of receptors for various bacterial adhesins (28), including HBHA, a surface-exposed glycosylated heparin-binding adhesin produced by \textit{M. tuberculosis} (15, 16).

For most adhesins, the molecular details of their interaction with GAG chains are not known. Here, we show that the carboxyl terminus of HBHA, which comprises five lysine-rich repeats (3 R1 and 2 R2), is responsible for heparin interaction. Consistent with the notion that basic amino acids are most often involved in sulfated polysaccharide binding via electrostatic interactions (29), a recombinant HBHA variant from which all the repeats were deleted was unable to bind to heparin. Progressive truncations within the HBHA repeats correlated with a reduction of affinity for heparin, and both the R1 and the R2 repeats were found to contribute to binding to the sulfated polysaccharide. When the repeats were grafted onto MBP, which by itself does not bind to heparin (30), the hybrid protein displayed the same affinity for heparin in real time (A). No interaction was observed with rHBHAC and MBP. Data are expressed as relative responses in resonance units (RU) after subtraction of the background signal recorded on streptavidin-coated sensor chips. The kinetic parameters of the interactions observed with rHBHA and MBP+3R1+2R2 are summarized in B. Standard deviations were calculated from three independent experiments.
the production of recombinant proteins easy to be purified by heparin-Sepharose chromatography. This application may have an advantage over other tags, such as histidine or protein A tags, in that the hybrid proteins can be easily eluted under mild, nondenaturing conditions, such as increased NaCl concentrations, whereas the elution of recombinant proteins tagged by other means usually requires urea or low pH (31, 32). Furthermore, the lysine-rich repeats are highly susceptible to proteolytic degradation (16), which should facilitate the removal of the heparin-binding tag after purification of the recombinant hybrid protein. However, the presence of other heparin-binding proteins in the recombinant cell extracts may require additional steps to purify the tagged protein.

SPR analysis of rHBHA-heparin interactions revealed high affinity binding, with affinity constants in the range of values that have been reported for the GAG-binding activities of eukaryotic proteins such as fibronectin (33), antithrombin III (34), or HARP, a heparin-binding growth factor (35). The affinity of HBHA for sulfated polysaccharides is thus sufficient to mediate adherence to the surface of physiologically relevant tissues, such as the pulmonary alveolus. By comparing different heparin-binding domains of various eukaryotic proteins,
Cardin and Weintraub (36) proposed two heparin-binding motifs, XBBXBB and XBBBXXB, where B represent basic amino acids and X any other amino acids. The heparin-binding site of HBHA does not totally fit the consensus sequence of Cardin and Weintraub (36). However, the high density of lysine residues within these repeats is consistent with their role as basic amino acids interacting with the negatively charged sulfate groups on the carbohydrate receptors (29). It is striking to notice that the HBHA heparin-binding domain is composed of only three amino acids (alanine, lysine, and proline) and consists on seven di-lysine motifs separated by the tetrapeptide AAPA or the tripeptides AAA or APA, which probably serve as spacers of the positive charges borne by the lysines.

The presence of proline-rich regions and repeats is known to induce aberrant electrophoretic mobility during SDS-PAGE. We have previously noticed that rHBHA displays such aberrant electrophoretic mobility (16). However, rHBHAAC still migrates slower than expected, indicating that the HBHA repeats are not responsible for its aberrant electrophoretic mobility.

The epitope recognized by mAb 3921E4 mapped within the lysine-rich repeats. This mAb has been reported to inhibit adherence of BCG to Chinese hamster ovary cells (15). We show here that this epitope comprises the sequence AAPAK-KAA. Deletion of this octapeptide from the HBHA protein abolished mAb 3921E4 recognition, and grafting of a peptide containing this octapeptide onto MBP resulted in the recognition of the recombinant hybrid protein by the mAb. Antibody-mediated blocking of mycobacterial binding to target cells may perhaps constitute the basis of a novel vaccine design against mycobacteria-host interactions. Given the high yield of rHBHA and the possibility to graft the heparin-binding site onto MBP, mycobacteria-host interactions are now feasible.

A previous study described for N. gonorrhoeae (39) and Chlamydia trachomatis (38) and Neisseria gonorrhoeae (39). Similar to this study, N. gonorrhoeae has been shown to adhere to heparan sulfate chains on epithelial cells, whereas chondroitin sulfate chains did not appear to be involved in gonococcal adherence (39). The purification and characterization of the HBHA receptor on the surface of pneumocytes will generate new valuable information on the mycobacteria-host interactions. Given the high yield of rHBHA and the possibility to graft the heparin-binding site onto MBP, as reported in this study, such investigations are now feasible.

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