Novel Mycobacteria Antigen 85 Complex Binding Motif on Fibronectin

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The members of the antigen 85 protein family (Ag85), consisting of members Ag85A, Ag85B, and Ag85C, are the predominantly secreted proteins of mycobacteria and possess the ability to specifically interact with fibronectin (Fn). Because Fn-binding proteins are likely to be important virulence factors of Mycobacterium spp., Ag85 may contribute to the adherence, invasion, and dissemination of organisms in host tissue. In this study, we reported the Fn binding affinity of Ag85A, Ag85B, and Ag85C from Mycobacterium avium subsp. paratuberculosis (MAP) (Kd values were determined from 33.6 to 68.4 nM) and mapped the Ag85-binding motifs of Fn. Fn14, a type III module located on the heparin-binding domain II (Hep-2) of Fn, was discovered to interact with Ag85 from MAP. The peptide inhibition assay subsequently demonstrated that a peptide consisting of residues 17–26 from Fn14 (SLLVSWQPPR26, termed P17–26) could interfere with Ag85 binding to Fn (73.3% reduction). In addition, single alanine substitutions along the sequence of P17–26 revealed that the key residues involved in Ag85-Fn binding likely contribute through hydrophobic and charge interactions. Moreover, binding of Ag85 on Fn siRNA-transfected Caco2 cells was dramatically reduced (44.6%), implying the physiological significance of the Ag85-Fn interaction between mycobacteria and host cells during infection. Our results indicate that Ag85 binds to Fn at a novel motif and plays a critical role in mycobacteria adherence to host cells by initiating infection. Ag85 might serve as an important colonization factor potentially contributing to mycobacterial virulence.

Background: The Ag85 proteins are fibronectin-binding proteins of mycobacteria.

Results: The binding site is mapped in the C terminus of fibronectin, Fn14.

Conclusion: Mycobacterial Ag85 proteins interact with the C terminus of fibronectin.

Significance: This was the first evidence that Ag85 proteins bind to the C terminus of fibronectin (Fn14).

The members of the antigen 85 protein family (Ag85), consisting of members Ag85A, Ag85B, and Ag85C, are the predominantly secreted proteins of mycobacteria and possess the ability to specifically interact with fibronectin (Fn). Because Fn-binding proteins are likely to be important virulence factors of Mycobacterium spp., Ag85 may contribute to the adherence, invasion, and dissemination of organisms in host tissue. In this study, we reported the Fn binding affinity of Ag85A, Ag85B, and Ag85C from Mycobacterium avium subsp. paratuberculosis (MAP) (Kd values were determined from 33.6 to 68.4 nM) and mapped the Ag85-binding motifs of Fn. Fn14, a type III module located on the heparin-binding domain II (Hep-2) of Fn, was discovered to interact with Ag85 from MAP. The peptide inhibition assay subsequently demonstrated that a peptide consisting of residues 17–26 from Fn14 (SLLVSWQPPR26, termed P17–26) could interfere with Ag85 binding to Fn (73.3% reduction). In addition, single alanine substitutions along the sequence of P17–26 revealed that the key residues involved in Ag85-Fn binding likely contribute through hydrophobic and charge interactions. Moreover, binding of Ag85 on Fn siRNA-transfected Caco2 cells was dramatically reduced (44.6%), implying the physiological significance of the Ag85-Fn interaction between mycobacteria and host cells during infection. Our results indicate that Ag85 binds to Fn at a novel motif and plays a critical role in mycobacteria adherence to host cells by initiating infection. Ag85 might serve as an important colonization factor potentially contributing to mycobacterial virulence.

Joehne’s disease occurs worldwide as a chronic intestinal disease of domestic and wild ruminants that is caused by Mycobacterium paratuberculosis (MAP)2 (1). The disease is characterized clinically by intermittent to persistent diarrhea, progressive weight loss, and eventually death (2). Early studies, based on cultures of ileocecal lymph nodes collected at slaughter, indicate that the prevalence of MAP in culled dairy cows is 2.9% (3). In addition to the agricultural economic impact, MAP has been suspected as a causative agent in Crohn disease in humans (4).

Microbial infections are initiated by molecular interactions between the pathogen and receptor molecules on host cells, resulting in microbial adhesion and, sometimes, subsequent internalization (5). Furthermore, when bacteria adhere to surfaces, they are substantially more resistant to host antimicrobial defenses (6). The extracellular matrix (ECM) of the cell consists of a complex mixture of macromolecules, including collagens, fibronectin, fibrinogen, vitronectin, laminin, and heparin sulfate (6), all of which function as ligands for bacterial adhesion. Fibronectin (Fn), a 220-kDa ECM protein that forms a dimer by disulfide linkage, is composed of three different modules subdivided into several different domains, including an N-terminal domain (NTD), a gelatin-binding domain (GBD), a cell-binding domain (CBD), a 40-kDa domain containing heparin-binding domain II (Hep-2, including Fn12, Fn13, and Fn14) and Fn15, and a fibrin-binding domain II (7, 8). Fn plays a pivotal role in bacteria-host interactions by interacting with microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) (9), a group of microbial surface proteins that interact with ECMs of host cells and initiate infection. MSCRAMMs that can bind to the NTD, the GBD (10–12), and the Hep-2 have been identified (13, 14).

Antigen 85 proteins (Ag85), consisting of members Ag85A, Ag85B, and Ag85C, sharing high sequence and structural homology are secreted and retained in the cell wall of mycobacteria (15). Ag85 expression is essential for intracellular survival of Mycobacterium tuberculosis within macrophage-like cell line models and is therefore likely to be a virulence factor (16). In addition to promoting excellent immunogenicity (17–20),
Ag85 possesses mycolyltransferase activity and catalyzes the synthesis of the most abundant glycolipid of the mycobacterial cell wall, trehalose 6,6-dimycolate (21). Ag85 interacts with Fn (22) at a specific Fn-binding motif (23). The Fn-binding motif of Ag85B of *Mycobacterium kansasii* has been reported previously to contain 11 residues, 99FEWYYQSGSLV108 (23). This motif has high homology to Ag85A and Ag85C (Fig. 1A) (24, 25, 36). In the context of the homologous Ag85 structures from *M. tuberculosis* (Fig. 1B), the Fn-binding motif includes a partial β-sheet and α-helix connected by a turn (24, 25). The helical region of the motif, 98FEWYYQ103, was demonstrated to be the most important for Fn binding (23) and also contains residues present on the surface of Ag85. The key negatively charged residue Glu99, the polar residue Gin103, and the hydrophobic residues Phe98, Trp100, Tyr101, and Tyr102 suggested that the corresponding binding site on Fn is likely to be composed of electrostatic and hydrophobic components.

In this study, the binding of Ag85 to Fn was characterized and localized to the Fn14 fragment of Fn. Furthermore, the Ag85-binding residues of Fn were determined to be SLLVSWQPPR. Finally, the binding of Ag85 to Fn was significantly reduced on Fn siRNA-transfected Caco-2 cells, indicating that Ag85 proteins are important Fn-binding antigens participating in mycobacterial invasion.

**MATERIALS AND METHODS**

**Bacterial Strains and Cell Culture—*Mycobacterium avium* subsp. *paratuberculosis* (MAP) strain K-10 was propagated in Middlebrook 7H9 (broth) and 7H10 (agar plate) (BD Biosciences). For medium preparation, 7H9 (4.7 mg) or 7H10 (19 mg) was supplemented with 10% oleic acid/albumin/dextrose/catalase (BD Biosciences), 0.05% Tween 80 (Sigma), 2.5 μg of mycobactin J (Allied Monitor, Fayetteville, MO), and glycerol (0.125 and 0.5%, for 7H9 broth and 7H10 agar plate, respectively) in tissue culture flasks. Caco-2 cells were cultured in Dulbecco’s minimum essential medium (DMEM) containing 10% fetal bovine serum (Invitrogen) and were grown at 37 °C in a humidified atmosphere with 5% CO2 (26). *Escherichia coli* strains were cultured in Luria-Bertani broth (LB) with appropriate antibiotics (Table 1).

**Reagents and Antibodies—**Fibronectin (extracted from human plasma), the N-terminal domain of Fn (NTD), the gelatin-binding domain of Fn (GBD), EDTA, sodium chloride, sodium phosphate monobasic, sodium phosphate dibasic, Tris, magnesium chloride, manganese chloride, zinc chloride, copper chloride, and calcium chloride were purchased from Sigma. The Fn cell-binding domain (CBD), the Fn 40-kDa domain, and mouse anti-fibronectin were ordered from Millipore (Billerica, MA). Mouse anti-α-actin antibody, mouse anti-histidine tag, mouse anti-GST antibody, and HRP-conjugated goat anti-mouse antibody were purchased from Millipore (Billerica, MA). Mouse anti-α-actin antibody, mouse anti-histidine tag, mouse anti-GST antibody, and HRP-conjugated goat anti-mouse antibody were purchased from Invitrogen. HRP-conjugated goat anti-mouse IgG antibody and 3,3',5,5'-tetramethylbenzidine (TMB) peroxidase substrate were purchased from Kirkegaard & Perry Laboratories (Gaithersburg, MD). Polyclonal *M. tuberculosis* Ag85 antibody was purchased from Mycobacterial Research Laboratories, Department of Microbiology, Immunology, Pathology, Colorado State University. Synthesized peptides (Table 2) were ordered from Genemed Synthesis (San Antonio, TX).

**Plasmid Construction and Protein Purification—**His-tagged MAP85A, Ag85B, and Ag85C were constructed by using the pET-16b vector (Merck). GST-tagged Fn12, Fn13, and Fn15 were generated using the pGEX-4T-2 vector (GE Healthcare). The oligonucleotides used for PCR were shown in Table 3. The recombinant protein plasmids were transformed into *E. coli* DH5α competent cells and then were streaked on a Luria-Bertani (LB) agar plate containing 100 μg/ml ampicillin.

### TABLE 1

| Strain or plasmid | Genotype or characteristic | Sources |
|------------------|---------------------------|---------|
| *M. avium* subsp. *paratuberculosis* K-10 | Wild type MAP strain | Invitrogen |
| *E. coli* strains | | |
| DH5α | | Promega |
| BL21 | | |
| pGEX4T-2 | Amp′, GST tag protein expression vector | GE Healthcare |
| pET-16b | Amp′, His tag protein expression vector | Merck |
| pGEX4T-2-Fn12 | pGEX4T2 carrying Fn12 | |
| pGEX4T-2-Fn13 | pGEX4T2 carrying Fn13 | |
| pGEX4T-2-Fn14 | pGEX4T2 carrying Fn14 | |
| pGEX4T-2-Fn15 | pGEX4T2 carrying Fn15 | |
| pET-16b-85A | pET-16b carrying MAP Ag85A | |
| pET-16b-85B | pET-16b carrying MAP Ag85B | |
| pET-16b-85C | pET-16b carrying MAP Ag85C | |

### TABLE 2

| Peptide | Sequence |
|---------|----------|
| P1−16 | AIDAPSNLRFLATTPN |
| P14−26 | TPNLSSLVSWQPPR |
| P25−38 | PRAITGYIIYRK |
| P37−52 | EKGPEVTATPGILPPP |
| P51−66 | EPGTEYTIYVIALKNN |
| P63−78 | EPQTVETATPGILPPP |
| P77−90 | NNOQSEPIRKKT |
| P17−26 | SSLVSWQPPR |
| P18−26 | SSLVSWQPPR |
| P19−25 | SSLVSWQPPR |
| P27−25 | SSLVSWQPPR |
| P35−41 | SSLVSWQPPR |
| P54−57 | SSLVSWQPPR |
| P55−58 | SSLVSWQPPR |
| P65−68 | SSLVSWQPPR |
| P57−60 | SSLVSWQPPR |
| P58−61 | SSLVSWQPPR |
| P59−62 | SSLVSWQPPR |
| P510−62 | SSLVSWQPPA |

*a Substituted residues are underlined.*
Colonies were selected from the agar plate and grown in 5 ml of LB culture containing 100 μg/ml ampicillin at 37 °C overnight. The correct construct was subsequently transformed into E. coli BL21 for protein expression. The 10-ml overnight culture of a single transformant was used to inoculate 1 liter of fresh LB medium containing 100 μg/ml ampicillin. The cells were grown to an A600 of 0.6 and induced with 1 mM isopropyl β-thiogalactopyranoside. After a 16-h post-induction growth at 20 °C, the cells were harvested by centrifugation at 6000 rpm for 15 min. The protein purification was conducted at 4 °C. The cell paste obtained from 1 liter of cell culture was suspended in 50 ml of phosphate-buffered saline (PBS). Cells were disrupted with a French press instrument (AIM-AMINCO Spectronic Instruments) applied at 12,000 p.s.i. The lysis solution was centrifuged (10,000 rpm for 30 min), and the pellet was discarded. The cell-free extract was loaded onto a 10-ml nickel-nitrilotriacetic acid-Sepharose column equilibrated with PBS. A 20-μl aliquot of His-tagged MAP Ag85 was added to each well and incubated at 37 °C for 1 h. Mouse anti-GST (1:1000) and HRP-conjugated goat anti-mouse IgG (1:1000) were used as primary and secondary antibodies, respectively. After washing the plates three times with PBST, 100 μl of TMB substrate (Kirkegaard & Perry Laboratories) was added to each well, allowed to react for 5 min, and quenched with the addition of 100 μl of 0.1% hydrochloric acid. The microtiter plates were read at 450 nm using an ELISA plate reader (Biotek EL-312). Each value represents the mean ± S.E. of three trials in triplicate samples.

**Surface Plasmon Resonance (SPR)**—The interactions of Fn to MAP Ag85 and Fn14 or P17–26 to MAP Ag85B were analyzed by an SPR technique using a Biacore 2000 instrument (GE Healthcare). To determine the Fn binding activity of Ag85 complex proteins, Fn (50 μg) was immobilized on a CM5 chip (GE Healthcare). Then 10 μl of MAP Ag85A, Ag85B, or Ag85C (in the concentration of 0, 2.8, 11.2, 22.5, 22.5, 45, and 90 nM) was injected into the flow cell at 10 μl/min at 25 °C. To measure the binding affinities of Fn14 and P17–26 to MAP Ag85B, MAP Ag85B was immobilized on a CM5 chip. A control flow cell was injected with PBS buffer without Fn14 or P17–26. The 10 μl of Fn14 (in the concentration of 0, 62.5, 250, 500, 1000, and 2000 nM) or P17–26 (0, 0.625, 2.5, 5, 10, and 20 μM) was injected into the flow cell at 10 μl/min at 25 °C. The chip surface was regenerated by removal of analyte with a regeneration buffer (10 mM glycine-HCl at pH 2.0). All sensogram data were subtracted from the negative control flow cell. To obtain the kinetic parameters of the interaction, the data of the sensograms were fitted by BIAevaluation software version 3.0 using the one-step biomolecular association reaction model (1:1 Langmuir model), which resulted in optimum mathematical fits with the lowest χ values.

**GST Pulldown Assay**—The GST pulldown assay was performed as described previously (30). Purified GST-fused Fn fragments (Fn12–15) or GST (negative control) was loaded onto 0.5 ml of glutathione-Sepharose beads (Amersham Biosciences) at 4 °C overnight. The beads were then washed three times with PBS. A 20-μl aliquot of His-tagged MAP Ag85B was incubated with GST-fused proteins immobilized on glutathione-Sepharose beads at 4 °C for 3 h. After incubation, the beads were separated by centrifugation, washed three times with PBS, and boiled in Laemmli sample loading buffer consisting of 50 mM Tris-HCl (pH 6.8), 100 mM dithiothreitol, 2% SDS, 0.25 mM PMSF, and 0.1% bromphenol blue in 20% glycerol. The eluted

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**Ag85-Fn Interaction**

**TABLE 3**

| Primer         | Sequence (5′ → 3′) |
|---------------|-------------------|
| MAP Ag85A Ndel-fp | GGAATTCATATGGCCGCAGTCGCTCGG |
| MAP Ag85A BamHI-rp  | CCGAATCTAGGGCGCCGAGGGCGCG |
| MAP Ag85B Ndel-fp  | CCGAATTCATATGGGCAGGCAGGGCG |
| MAP Ag85B Xhol-rp   | CCGAATTCATATGGGCAGGCAGGGCG |
| MAP Ag85C Ndel-fp  | CCGAATTCATATGGGCAGGCAGGGCG |
| MAP Ag85C BamHI-rp  | CCGAATTCATATGGGCAGGCAGGGCG |
| Fn12 EcoRI-fp      | CCGAATTCATATGGGCAGGCAGGGCG |
| Fn12 Xhol-rp       | CCGAATTCATATGGGCAGGCAGGGCG |
| Fn13 EcoRI-fp      | CCGAATTCATATGGGCAGGCAGGGCG |
| Fn13 Xhol-rp       | CCGAATTCATATGGGCAGGCAGGGCG |
| Fn14 EcoRI-fp      | CCGAATTCATATGGGCAGGCAGGGCG |
| Fn14 Xhol-rp       | CCGAATTCATATGGGCAGGCAGGGCG |
| Fn15 EcoRI-fp      | CCGAATTCATATGGGCAGGCAGGGCG |
| Fn15 Xhol-rp       | CCGAATTCATATGGGCAGGCAGGGCG |

**Notes:**

- The primer sequences are designed to amplify the desired fragments from the full-length Fn sequence. The restriction enzyme sites are underlined. The oligonucleotides used in this study are listed in Table 3.

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**Protein Binding Assays by ELISA**—To measure the binding affinity of MAP Ag85 complex proteins, Fn (50 μg) was immobilized on a CM5 chip (GE Healthcare). Then 10 μl of MAP Ag85A, Ag85B, or Ag85C (in the concentration of 0, 2.8, 11.2, 22.5, 22.5, 45, and 90 nM) was injected into the flow cell at 10 μl/min at 25 °C. To measure the binding affinities of Fn14 and P17–26 to MAP Ag85B, MAP Ag85B was immobilized on a CM5 chip. A control flow cell was injected with PBS buffer without Fn14 or P17–26. The 10 μl of Fn14 (in the concentration of 0, 62.5, 250, 500, 1000, and 2000 nM) or P17–26 (0, 0.625, 2.5, 5, 10, and 20 μM) was injected into the flow cell at 10 μl/min at 25 °C. The chip surface was regenerated by removal of analyte with a regeneration buffer (10 mM glycine-HCl at pH 2.0). All sensogram data were subtracted from the negative control flow cell. To obtain the kinetic parameters of the interaction, the data of the sensograms were fitted by BIAevaluation software version 3.0 using the one-step biomolecular association reaction model (1:1 Langmuir model), which resulted in optimum mathematical fits with the lowest χ values.

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**Oligonucleotides used in this study**

| Primer         | Sequence (5′ → 3′) |
|---------------|-------------------|
| MAP Ag85A Ndel-fp | GGAATTCATATGGCCGCAGTCGCTCGG |
| MAP Ag85A BamHI-rp  | CCGAATCTAGGGCGCCGAGGGCGCG |
| MAP Ag85B Ndel-fp  | CCGAATTCATATGGGCAGGCAGGGCG |
| MAP Ag85B Xhol-rp   | CCGAATTCATATGGGCAGGCAGGGCG |
| MAP Ag85C Ndel-fp  | CCGAATTCATATGGGCAGGCAGGGCG |
| MAP Ag85C BamHI-rp  | CCGAATTCATATGGGCAGGCAGGGCG |
| Fn12 EcoRI-fp      | CCGAATTCATATGGGCAGGCAGGGCG |
| Fn12 Xhol-rp       | CCGAATTCATATGGGCAGGCAGGGCG |
| Fn13 EcoRI-fp      | CCGAATTCATATGGGCAGGCAGGGCG |
| Fn13 Xhol-rp       | CCGAATTCATATGGGCAGGCAGGGCG |
| Fn14 EcoRI-fp      | CCGAATTCATATGGGCAGGCAGGGCG |
| Fn14 Xhol-rp       | CCGAATTCATATGGGCAGGCAGGGCG |
| Fn15 EcoRI-fp      | CCGAATTCATATGGGCAGGCAGGGCG |
| Fn15 Xhol-rp       | CCGAATTCATATGGGCAGGCAGGGCG |
proteins were subjected to 12% SDS-PAGE and electroblotted onto polyvinylidene difluoride membranes. The membranes were incubated in 5% skim milk in PBST overnight and then incubated with mouse anti-histidine antibody (1:1000). The immunocomplexes were detected by HRP-conjugated goat anti-mouse IgG antibody (1:5000).

**Peptide Inhibition Assay**—Fn (0.5 μg) was coated on microtiter plate wells and blocked as described previously. His-tagged MAP Ag85B (0.1 μM) was preincubated with a 50 μM concentration of synthetic peptides in 100 μl of PBS buffer at 4 °C for 1 h. The MAP Ag85B/peptide mixture was then added directly to Fn-coated microtiter wells. Binding was detected by ELISA as described above.

**Small Interfering RNA (siRNA) Inhibition of MAP Ag85B Binding**—Synthetic siRNA duplexes directed against human Fn (AM11012) and negative siRNA duplex (AM4611) were purchased from Applied Biosystems (Carlsbad, CA). RNA duplexes were introduced into Caco-2 cells by the method of lipofection. The knockdown efficiency of MAP Ag85B (0.1 M) was preincubated with a 50 μM concentration of synthetic peptides in 100 μl of PBS buffer at 4 °C for 1 h. The MAP Ag85B/peptide mixture was then added directly to Fn-coated microtiter wells. Binding was detected by ELISA as described above.

**Bacterial and Protein Attachment Assays**—To investigate the adhesion of MAP strain K-10 or recombinant MAP Ag85B on Caco-2 cells, the attachment assay was slightly modified from the method reported previously (33). Basically, Caco-2 cells (10⁶) were cultured on microtiter plate wells at 37 °C overnight. Ice-cold PBS buffer was used to wash the cells prior to the assays to prevent MAP K-10 invasion. For bacterial attachment assay, a total of 1.4 × 10⁵ MAP K-10 was suspended in ice-cold medium and then incubated with coated Caco-2 cells at 4 °C for 1 h. The wells incubated without Caco-2 cells served as negative controls. Unattached bacteria were removed by five washes with PBS. PBS containing 1% (v/v) Triton X-100 was applied to resuspend adherent bacteria, and the 10²-fold dilutions of adherent bacterial suspension were spread on 7H10 agar plates to determine the number of cell-associated bacteria per well. For the protein attachment assay, filtered (0.45 μm) His-tagged MAP Ag85B (0.1 μM) was added to the wells coated with Caco-2 cells (10⁶) and incubated at 37 °C for 1 h. Wells were washed three times with PBS. Bound MAP Ag85B was detected as mentioned above.

**Protein Depiction**—Images of protein structures were generated using UCSF Chimera (35). The surface-accessible surface areas for residues were also computed using UCSF Chimera.

**RESULTS**

**Binding Affinity of MAP Ag85 to Fn**—Because of the slight variation among the Fn-binding motifs of Ag85 (Fig. 1, A and B), differences in the $K_D$ values were measured as 68.4 ± 4.6 nM (MAP Ag85A), 36.7 ± 5.4 nM (MAP Ag85B), and 33.6 ± 4.2 nM (MAP Ag85C), respectively (Fig. 1C). The $k_{on}$, $k_{off}$, and $K_D$ values are summarized in Table 4. Unlike the interaction of some bacterial adhesins and ECM components (32, 34, 37), divalent metal ions (Ca²⁺, Cu²⁺, Co²⁺, Fe²⁺, Mg²⁺, Mn²⁺, Ni²⁺, and Zn²⁺) were not required for the Ag85-Fn interaction (data not shown).

**MAP Ag85B Binds to 40-kDa Domain of Fn**—To identify the MAP Ag85B-binding fragments on Fn, NTD, GBD, CBD, and the 40-kDa domains of Fn (Fig. 2A) were examined by using ELISA. As shown on Fig. 2B, the 40-kDa domain was the only domain to strongly interact with MAP Ag85B. No significant interaction between MAP Ag85B and the NTD, GBD, or CBD was observed.

**Fn14 Possesses MAP Ag85B-binding Activity**—The 40-kDa domain of Fn was subsequently divided into four fragments (Fn12, Fn13, Fn14, and Fn15), and each was fused with GST tags (Fig. 3A). As presented in Fig. 3A, Fn14 significantly bound to immobilized MAP Ag85B, whereas Fn12, Fn13, or Fn15 could not bind. The binding potency ($K_D$) of Fn14 to MAP Ag85B measured by SPR was 48.1 ± 4.7 nM (Fig. 3B). GST pulldown assay further demonstrated that Fn14 was the solitary fragment binding to MAP Ag85B (Fig. 3C). Moreover, Fn14-pretreated MAP Ag85B apparently diminished its affinity to interact with Fn, indicating that MAP Ag85B bound to Fn by targeting to Fn14 (Fig. 3D).

**Mapping the Ag85-binding Motif on Fn14**—Seven peptides synthesized based on the amino acid sequence of Fn14 were subjected to the peptide inhibition assay to identify the Ag85-binding motif (Fig. 4A). As shown on Fig. 4B, Fn14 residues 14–26, termed P14–26 (14TPNSLLVSWQPPR26), harbored the highest inhibition potency among those peptides. Fn14 residues 17–26, termed P17–26 (17SLLVSWQPPR26), were derived from P14–26 with a three-amino acid truncation at the C terminus (18LLVSWQPPR26) or the N terminus (15TPNSLLVSWQPPR26), which lost most of the ability to interfere with MAP Ag85B-Fn binding (Fig. 4C). Peptides with an additional one-amino acid deletion from either the N terminus (18TPNSLLVSWQPPR26) or the C terminus (17TPNSLLVSWQPPR26) lost most of the ability to interfere with MAP Ag85B-Fn binding (Fig. 4C). The measured $K_D$ value of P17–26 with MAP Ag85B was 414.2 ± 13.6 nM (Fig. 4D). As a result, the motif on Fn14 required for high affinity MAP Ag85B binding was SLLVSWSQPPR.

**Single Residue Substitution of P17–26**—Ten peptides (PS1–10) (Table 2) were derived from P17–26 by single alanine scanning substitution and were applied to determine the key binding residues. Analogous peptides were subjected to the peptide inhibition assay (Fig. 5A). Compared with P17–26, PS3 (L19A), PS4 (V20A), PS5 (S21A), PS7 (Q23A), PS8 (P24A), PS9 (P25A), and PS10 (R26A) showed dramatically reduced inhibition
potencies (>50% reduction). However, PS1 (S17A), PS2 (L18A), and PS8 (W22A) showed only minor inhibition of Ag85-Fn binding (20–25% reduction). The data are mapped onto the structure of Fn14 (Fig. 5B) (38). The 10 amino acids corresponding to P17–26 are a part of the second β-sheet and extend into a less structured loop containing adjacent proline residues.

Transfection of Fn siRNA Inhibits MAP Ag85B Binding on Mammalian Cells—To further elucidate the role of Fn as a cell surface receptor for the putative binding partner, Ag85, the binding of MAP Ag85B to Caco-2 cells was examined. Fn is normally expressed on the surface of Caco-2 cells (32). Fn expression levels can be reduced by 62.7% with transfected Fn siRNA duplex as compared with cells transfected with negative siRNA duplex (Fig. 6A). The binding of recombinant MAP Ag85B was 44.6% less for the Fn siRNA-transfected Caco-2 cells with greatly reduced Fn expression (Fig. 6B, solid bar).

However, only a 9.7% decreased binding in adhesion of MAP K-10, the wild-type MAP strain expressing Ag85 proteins (supplemental Fig. 1), to Fn siRNA-transfected Caco-2 cells was detected (Fig. 6B, open bars).

### DISCUSSION

Adhesion is a pivotal first step that allows pathogenic bacteria, including mycobacteria, to infect host cells. MSCRAMMs, a group of virulence factors located in the outer surface of bacteria, mediate adhesion of a wide variety of pathogenic bacteria, including *Staphylococcus*, *Streptococcus*, *Enterococcus*, *Borrelia*, Leptospira, and others to ECM components such as Fn, fibrinogen, collagen, elastin, laminin, and peptidoglycan (6, 9, 27, 31, 39–41). Ag85 proteins, including Ag85A, Ag85B, and Ag85C, have been reported to interact with Fn (15, 42). In addition to Ag85, a number of mycobacteria proteins were also identified to bind to Fn such as MPT51 (also called Ag85D or

### TABLE 4

| Fn-binding motif | $k_{on}$ | $k_{off}$ | $K_D$ |
|------------------|---------|---------|------|
| MAP Ag85A        | FEWYNQSGISV | $9.2 \times 10^6 \pm 1.1$ | $6.3 \times 10^{-3} \pm 0.8$ | 68.4 ± 4.6 |
| MAP Ag85B        | FEWYYQSGGLSV | $1.2 \times 10^6 \pm 0.2$ | $4.4 \times 10^{-3} \pm 0.7$ | 36.7 ± 5.4 |
| MAP Ag85C        | FEFQYGSLSV | $1.4 \times 10^6 \pm 0.3$ | $4.7 \times 10^{-3} \pm 0.7$ | 33.6 ± 4.2 |

*Data were reported by Naito et al. (23).*
FbpD) (43) and ModD (44, 45). Binding to Fn, likely a significant factor in the virulence of mycobacteria, represents a potential first step in the attachment and entry of mycobacteria into host cells.

The Fn-binding motif, a stretch of 11 residues (FEWYYQS-GLSV), of Ag85B from *M. kansasii* has been reported previously (23). In the three-dimensional structure of Ag85, the residues are located on the surface opposite the substrate and cell wall interacting region providing access for Fn binding while attached to mycobacteria (46). Binding differences between Fn-binding motifs from MAP Ag85A, Ag85B, and Ag85C could be explained by amino acid sequence variations. A hydrophobic residue (Tyr) of MAP Ag85B and Ag85C is a hydrophilic one (Asn) in the corresponding position of MAP Ag85A, resulting in a reduced affinity to Fn (~2-fold decrease). A similar Fn binding affinity was observed for MAP Ag85B and Ag85C, even though Glu and Phe are substituted for Trp and Tyr, respectively, in Ag85C. The Ag85 structure (24, 25) as well as a previous alanine substitution study (23) suggest that the FEWYYQ helix is the most likely region for direct Fn interaction (supplemental Fig. 2A). In this sequence, the 2nd position Glu, the 3rd position Trp, the 5th position Tyr, and the 6th position Gln are surface-accessible (Fig. 1B). Although the 2nd position Glu and the 6th position Gln are conserved among MAP Ag85 proteins, differences for 3rd and 5th position hydrophobic residues suggest that the 3rd position Trp is less likely to be involved in direct Fn binding than the 5th position Tyr. The residues involved provide a basis for both hydrophobic and charged interactions.

Here, we demonstrated that MAP Ag85B binds to the 40-kDa domain of Fn (Fig. 2B), and we further showed that Fn14 was the only fragment to independently interact with MAP Ag85B (KD = 48.1 ± 4.7 nM), with the similar binding affinity to that of Fn (KD = 36.7 ± 5.4 nM) (Fig. 3, A–D). Previous reports locating the Ag85-binding sites on Fn have differed as the GBD was suggested by Peake *et al.* (47) and the CBD and the Hep-2 were suggested by Naito *et al.* (48). The discrepancy might be because the recombinant *Mycobacterium bovis*
Ag85B was prepared under denaturing conditions for the studies conducted by Peake et al. (47), which might have caused the protein to lose its native folded structure. In the study by Naito et al. (48), the Ag85-binding fragments of Fn were identified by a proteolytic strategy (thermolysin digestion); however, following the proteolytic digestion, the real Ag85-binding fragment of Fn (SLLVSWQPPR) was possibly missed because thermolysin preferably cleaves substrates with bulky hydrophobic residues (Leu, Ile, Val, and Phe) at the P1 position (49). Moreover, the Ag85-binding site was mapped to Hep-2, a huge 30-kDa domain composed by Fn12, Fn13, and Fn14, using recombinant protein that locates a region but does not establish the specific binding site. Meanwhile, we demonstrated that the addition of heparin (100 nM) did not interfere with the binding between the 40-kDa domain (Fn12–15) and MAP Ag85B (data not shown), indicating that the Ag85-binding site on Fn is distinct from the heparin-binding sequence on Fn13. In this study, we divided the 40-kDa domain of Fn into four individual fragments (Fn12, Fn13, Fn14, and Fn15) and then tested each to further establish the specifics of binding. Fn14 was proven to be the MAP Ag85B-interacting fragment.

The Ag85-binding motif (17SLLVSWQPPR26) of Fn14 that we have identified is a typical β-strand in the type III Fn core structure (38). The hydrophobic residues (Leu19, Val20, Pro24, and Pro25), polar residues (Ser21 and Gln23), and positively charged residue (Arg26) in the Fn motif were essential for MAP Ag85B binding (Fig. 5A). Mapped onto the Fn14 structure, the Ag85-binding motif residues extend along the length of the domain (Fig. 5B). The most exposed region for a direct interaction with Ag85 is the stretch from Gln23 to Arg26 in Fn14. Positioning the structure of Fn14 near its binding site on Ag85, potential Fn14-Ag85 interactions exist to explain the experimental results (Fig. 7). Matching the negatively and positively charged residues on Ag85 and Fn14, respectively, a hydrophobic and hydrogen-bonding interaction would line up and match the Fn-interacting residues on Ag85. Because all Ag85 crystal structures are very similar overlaying structures for the Fn-binding region, the Fn-interacting helix should maintain its conformation upon interacting with Fn14 (supplemental Fig. 2, B and C). Although the potential Ag85-binding site is near the link between Fn14 and Fn13, the position of Fn13 in the Fn12–Fn14 crystal structure would not sterically inhibit Ag85 binding to Fn14. This docking site fails to explain the effect of mutations at positions Leu19 through Ser21, but because these residues are sandwiched between two other β-sheets, they are less exposed for Ag85 interactions. Also, the N-terminal end of the Ag85-binding region (17SLLVSW22) is more hydrophobic and therefore more likely to be involved in nonspecific binding interac-

FIGURE 3. Fn14 bound to MAP Ag85B. A, binding of fragments derived from the 40-kDa domain (including Fn12, Fn13, Fn14, and Fn15) to immobilized MAP Ag85B. GST-tagged Fn12, Fn13, Fn14, or Fn15 (0.1 μM) was added to microtiter plate wells coated with 0.5 μg of MAP Ag85B. Bound proteins were detected by ELISA. B, KD determination of Fn14 to MAP Ag85B by SPR analysis. The measured k_on, k_off, and KD values were $7.7 \times 10^3 \pm 1.8 \text{ M}^{-1} \text{s}^{-1}$, $3.7 \times 10^{-4} \pm 0.6 \text{ s}^{-1}$, and $48.1 \pm 4.7 \text{ nM}$, respectively. Values represent the mean ± S.E. C, His-tagged MAP Ag85B was applied to the GST beads preimmobilized by recombinant Fn12 (lane 1), Fn13 (lane 2), Fn14 (lane 3), Fn15 (lane 4), or GST (lane 5; negative control). The pulldown complex was analyzed by immunoblot analysis using anti-His antibody. D, Fn14 interfered with the binding of MAP Ag85B to Fn. MAP Ag85B was preincubated with various concentrations (0.05, 0.1, 0.2, 0.5, and 1 μM) of Fn12, Fn13, Fn14, or GST (negative control) prior to interaction with immobilized Fn. Bound proteins were detected by ELISA.
tions. In addition, two similar sequences were found in Fn2 (SFVVSWVSAS) and Fn10 (SLLISWDAPA) located within the CBD (data not shown), which might explain that CBD contributed a weak (~7.8% relative binding affinity) but not major affinity to MAP Ag85B binding, as well as the results from Naito et al. (23) (Fig. 2B). In agreement with a previous report (23), our positioning of the protein-protein interacting surface suggests that both a charge-charge (Glu-Arg) and hydrophobic interactions are required for Ag85-Fn interaction. Interestingly, the determined KD value of P17–26 (414.2 ± 13.6 nM) was significantly larger than that of Fn (36.7 ± 4.2 nM) or Fn14 (48.1 ± 4.7 nM). The three-dimensional structure and folding could be important for Fn binding to Ag85, because P17–26 is likely to be a structurally flexible peptide.

Binding to components of the ECM, including Fn, facilitates the adhesion of pathogens to host cells (50). Fn also serves as a mediator to induce endocytosis and initiate the entry of bacteria upon binding to bacterial surface proteins (51, 52). We tested whether reducing the expression of Fn on Caco-2 cells would impair the ability of Ag85 or Ag85-expressing MAP K-10 to bind to the Caco-2 cells. The reduced binding (44.6%) of MAP Ag85B to Fn siRNA-treated Caco-2 cells suggests an important adhesive role for the Ag85 protein family. In Fn siRNA-transfected Caco-2 cells, Fn expression remains at 37.3% of endogenous levels allowing MAP Ag85B to still bind but at a reduced level. The corresponding loss of Fn expression and MAP Ag85B binding suggests that Fn is the main cell surface receptor for MAP Ag85B. However, only a 9.7% reduction was observed in MAP K-10 binding to Fn siRNA-transfected Caco-2 cells. These results indicate that the Ag85-Fn interaction accounts for only a portion of the ability of a MAP cell to adhere to a host cell. Other surface antigens such as FbpD,
ModD, or unidentified proteins of MAP K-10 probably participate in additional ECM interactions (44, 45, 53–55).

In conclusion, we have characterized the binding affinities of Ag85 to Fn and demonstrated that MAP Ag85B interacts with Fn by targeting to a novel motif (SLLVSWQPPR). Our finding also clarified that the interaction mechanism between Ag85 proteins and Fn relies on residual charge and hydrophobic interactions. Because Ag85 proteins are important colonization factors potentially contributing to mycobacterial virulence and play not only a role in Fn-mediated host attachment but possess the essential acyltransferase activity contributing to the biosynthesis of mycobacterial cell wall, the Ag85 protein family is an important target for further antimycobacterial studies.

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