Characterization of the Fibronectin Binding Motif for a Unique Mycobacterial Fibronectin Attachment Protein, FAP*

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Studies were performed to define the fibronectin binding motif of the previously identified Mycobacterium avium fibronectin attachment protein (FAP-A). Using synthetic peptides of a previously identified fibronectin binding region (amino acids 269–292), the minimal binding sequence was determined to be 12 amino acids, 269–280 (FAP-A(269–280)). Synthetic peptides were prepared in which each amino acid in the 269–280 sequence was substituted with Ala. Assessment of the effect of Ala substitution on fibronectin binding showed that the presence of Ala at amino acids 273–276 (RWFV) completely abrogated fibronectin binding activity. Furthermore, the ability to inhibit the attachment of viable Mycobacterium bovis BCG to fibronectin was abrogated by Ala substitution at the RWFV sites. To validate the function of RWFV, further studies were performed with recombinant FAP-A in which single Ala mutations were generated for the RWFV sites and as controls at amino acids 269 and 280. Mutant FAP-A containing single Ala substitutions at the RWFV sites (amino acids 273, 274, 275, or 276) showed significant abrogation of fibronectin binding function. Recombinant FAP-A with Ala substitutions at either 269 or 280 showed wild type activity. When the four essential amino acids (RWFV) were either substituted en bloc with Ala or were all deleted, complete loss of fibronectin binding function was observed. Control recombinant proteins with en bloc Ala substitutions or deletions at four positions outside the fibronectin binding region (amino acids 255–257) retained functional activity. These data show that the RWFV sequence is necessary for fibronectin binding function of FAP-A. Furthermore, the data suggest that mycobacterial FAP proteins, all of which share the RWFV binding motif, constitute a family of highly homologous proteins that bind fibronectin in a unique manner.

The attachment of mycobacteria to fibronectin is well documented (1, 2). All tested species including Mycobacterium avium strain BCG, Mycobacterium tuberculosis, Mycobacterium kansasii, Mycobacterium avium, Mycobacterium leprae, and Mycobacterium smegmatis were observed to attach to fibronectin (3–5). In studies on M. bovis BCG-mediated immunotherapy, which is the treatment of choice for superficial bladder cancer, fibronectin attachment was shown to be necessary for the expression of antitumor activity (2). In addition, the attachment and internalization of M. bovis BCG, M. avium, and M. leprae by epithelial cells and Schwann cells also were shown to be dependent on bacterial attachment to fibronectin (4, 6, 7). Thus, an understanding of the interaction between mycobacterial proteins mediating attachment to fibronectin is needed.

Two distinct mycobacterial proteins or protein complexes, fibronectin attachment protein (FAP)1 and proteins of the antigen 85 complex, have been linked to mycobacterial attachment to fibronectin (8, 9). The interaction of proteins from the antigen 85 complex with fibronectin has been characterized using recombinant 85A, 85B, and 85C proteins (10–12). The best characterized of the complex is the 85B protein. Using in vitro assays, the binding of 85B to fibronectin was observed to depend on a FEWYYQ binding motif (12). This motif is highly conserved among all antigen 85 proteins. Characterization of the interaction of antigen 85 proteins with fibronectin has been limited to studies defining the binding of recombinant proteins to fibronectin. The role of antigen 85 proteins and the FEWYYQ sequence in binding of viable bacteria to fibronectin is not known.

FAP proteins constitute a family of highly homologous proteins of the mycobacteria. Polyclonal antibodies to purified Mycobacterium vaccae FAP (FAP-V) also recognize FAP proteins of M. leprae (FAP-L), M. avium (FAP-A), M. bovis BCG (FAP-B), M. kansasii, M. tuberculosis, and M. smegmatis (FAP-S) (4, 5, 7). To date, FAP proteins from four mycobacterial species including M. leprae (FAP-L), M. avium (FAP-A), M. bovis BCG (FAP-B), and M. smegmatis (FAP-S) have been cloned and characterized (4, 5).2 Using recombinant FAP-A and FAP-A peptides, two non-continuous fibronectin binding regions (amino acids 177–201, 235–257) were observed to possess fibronectin binding activity (5). Both sequences are highly conserved among all identified FAP proteins except FAP-S, which contains only the region homologous to FAP-A(269–292) (5). In vitro studies on FAP-A(269–292) or its homologue in M. leprae, FAP-L(240–263) showed this fibronectin binding region to be sufficient to block the attachment of all tested mycobacteria (M. avium, M. bovis BCG, and M. smegmatis) to fibronectin-coated surfaces and also to abrogate fibronectin-opsazonized mycobacterial attachment to epithelial cells and Schwann cells (5). These data suggest an important role for FAP-A(269–292) in FAP-mediated fibronectin binding.

In the present study, the amino acids required for fibronectin binding of the FAP-A(269–292) peptide were determined us-

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1 The abbreviations used are: FAP, fibronectin attachment protein; FN, fibronectin; LN, laminin.
2 W. Zhao, submitted for publication.
ing Ala substitutions in synthetic peptides and site-directed mutagenesis of the recombinant protein. The data show that four amino acids, RWFV, are necessary for fibronectin binding function.

EXPERIMENTAL PROCEDURES

Synthesis of FAP-A Peptides—The FAP-A peptides were synthesized and purified by high performance liquid chromatography as described (5).

Peptide-FN Binding Assay—The FN binding to FAP-A peptides was as described (5). Briefly, immulon 2 microtiter 96-well plates were coated with FAP peptides, 0.36 mM for Fig. 1 and the given concentration for Fig. 2. Nonspecific sites were blocked with bovine serum albumin, and 1 μg of FN or laminin (LN) in wash buffer (phosphate-buffered saline, 0.1% bovine serum albumin, 0.05% Tween 20) was added per well. After a 3-h incubation at 25°C, wells were washed with wash buffer and bound FN and LN were detected using rabbit polyclonal anti-FN or anti-LN, respectively. Bound antibody was detected using anti-rabbit Ig coupled to horseradish peroxidase.

Site-directed Mutagenesis of FAP-A Gene—Site-directed mutagenesis by overlap extension was used to generate mutant FAP-A DNA (13, 14). For each mutant FAP-A DNA, one pair of complementary oligonucleotide primers containing GCC (code for alanine) substitution or nucleotide deletion at the desired site were used to generate two DNA fragments having overlapping ends with primary polymerase chain reaction. Then these fragments were combined for secondary polymerase chain reaction. The resultant mutant FAP-A DNAs were cloned into pBluescript SK vector (Stratagene, La Jolla, CA) and sequenced using the Taq DyeDeoxy Termination Cycle Kit and the Applied Biosystems 373A DNA sequencer (Applied Biosystems, Foster City, CA).

Expression and Purification of FAP-A Mutants—All mutant FAP-A DNAs were digested with BamHI and EcoRI and then ligated into the expression vector pTrcHisB. The mutant FAP-A fusion proteins containing poly-His tag were expressed and purified using a Ni2+ affinity column under denaturing conditions according to the manufacturer’s protocol (Invitrogen, Carlsbad, CA). The cell lysates and purified fusion proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and visualized by Coomassie Blue staining.

In Vitro Attachment of M. bovis BCG to FN-coated Surface—M. bovis BCG attachment was performed as described previously (5). Immulon 4 microtiter plates (96-well) were coated overnight at 4°C with 100 μl of 60 μg/ml fibronectin or 120 μg/ml human serum albumin. After blocking nonspecific sites with human serum albumin, a total of 2 × 10^6 colony forming units of fluorescein isothiocyanate-labeled M. bovis BCG were added in a volume of 50 μl of 0.05 M Tris buffer (pH 6.2). After a 90-min incubation at 37°C, the wells were washed with 0.05 M Tris buffer and bound fluorescein isothiocyanate-labeled M. bovis BCG were detected using a FL500 Fluorescence Plate Reader (Bio-Tek Instruments, Frederick, MD).

RESULTS

Localization of the Binding Site on FAP-A—Previous studies identified two regions in FAP-A that possessed the capacity to bind fibronectin (5). These regions consisted of amino acids...
177–201 (FAP-A-(177–201)) and amino acids 269–292 (FAP-A-(269–292)). Characterization of the peptides showed that FAP-A-(269–292) was sufficient to block the interaction of mycobacteria with fibronectin-coated surfaces, mycobacterial attachment to epithelial cells, and *M. bovis* BCG-mediated antitumor activity (4, 5). Therefore we initiated studies on FAP-A-(269–292) to determine the amino acids required for fibronectin binding. Initially we tested synthetic peptides of varying lengths to determine the minimal peptide length necessary for binding to fibronectin. Truncation of FAP-A-(269–292) from the amino-terminal end abrogated binding activity quickly suggesting that this region played an important role in fibronectin binding (Fig. 1A). Truncation from the carboxyl-terminal end showed that the minimal peptide length supporting fibronectin binding was amino acids 269–280 (FAP-A-(269–280), Fig. 1B). No binding function was observed for a peptide containing amino acids 269–277.

Further studies were performed to identify the essential amino acids within FAP-A-(269–292). To determine the essential amino acids, peptides were synthesized that contained single Ala substitutions as shown in Fig. 2A. Each peptide was tested for binding capacity to fibronectin as described under “Experimental Procedures” (Fig. 2). The data show that amino acids 273–276 are essential for fibronectin binding (Fig. 2, B and C). Ala substitution of any one of these amino acids completely abrogated fibronectin binding activity. Substitution of amino acids 269–272, 277, and 279 had no effect on the fibronectin binding capacity of FAP-A-(269–280) (Fig. 2C). Ala substitution of amino acid 278 produced partial inhibition of binding and amino acid 280 reproducibly enhanced the fibronectin binding capacity of the FAP-A-(269–280) peptide (Fig. 2C). Comparison of binding regions for cloned FAP proteins shows 100% homology among the essential amino acids (RWFV; Fig. 2D).

**Effect of Synthetic Peptides with Ala Substitution on M. bovis BCG Attachment to Fibronectin**—We previously reported the ability of the synthetic FAP-A-(269–292) peptide to inhibit the attachment of *M. bovis* BCG to fibronectin-coated surfaces (5). Here we tested the wild type minimal binding peptide (FAP-A-(269–280)) and FAP-A-(269–280) with individual Ala substitution (at amino acid 269, 273, 274, 275, 276, or 280) for their ability to inhibit *M. bovis* BCG attachment to fibronectin. When compared with wild type peptide (FAP-A-(269–292)), peptides with Ala substitution within the active region identi-
fied above either partially (Ala$^{274-276}$) or completely (Ala$^{273}$) abrogated the ability of the respective peptides to block *M. bovis* BCG attachment (Fig. 3, A and B). The relative activity of peptides with Ala substitutions at 274–276 varied from experiment to experiment although the data consistently showed partial abrogation of activity for each. Therefore, we show the mean percent inhibition of all experiments (three in number) in Fig. 3B. These data show the reproducibility of the effects of Ala substitution. In all experiments control peptides containing Ala substitution at either amino acid 269 or 280 had no significant effect on peptide function (Fig. 3, A and B). 

**Effect of Site-directed Mutagenesis of FAP-A on *M. bovis* BCG Attachment to Fibronectin**—To determine the effect of Ala substitution on fibronectin binding activity at the FAP-A fusion protein level, a truncated form of FAP-A consisting of amino acids 210–381 (FAP-A-(210–381)), which contains the FAP-A-(269–292) binding region, was used. Residues 269, 273, 274, 275, 276, and 280 were selected for individual Ala substitution (Fig. 4A). In addition the four identified active amino acids, RWFV (amino acids 273–276), were either deleted en bloc or substituted en bloc with Ala. As a control for the RWFV deletion and the en bloc Ala substitution, four residues outside the identified fibronectin binding region, NGQI (amino acids 255–257), were either deleted or substituted with Ala. The sequences of all mutated FAP-A DNAs were confirmed by DNA sequencing. FAP-A mutants were subcloned into the pTrcHis expression vector and expressed, and the resultant fusion proteins were purified as described (4, 5). Coomassie Blue staining showed the induced fusion proteins to correspond to the correct molecular weight (data not shown).

The effect of mutant FAP-A proteins on *M. bovis* BCG attachment to fibronectin was tested, and a representative experiment showing *M. bovis* BCG attachment is provided in Fig. 4B. Fig. 4C shows the mean percent inhibition from three independent experiments of *M. bovis* BCG binding by the FAP-A mutants. Mutant FAP-A with a single Ala substitution at either amino acid 269 (FAP-A$^{269}$) or 280 (FAP-A$^{280}$) showed no alteration in peptide function (Fig. 4, B and C). In contrast FAP-A mutants with Ala substitutions at amino acids 273 (FAP-A$^{273}$), 274 (FAP-A$^{274}$), 275 (FAP-A$^{275}$), and 276 (FAP-

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**Fig. 3.** Inhibition of *M. bovis* BCG attachment to fibronectin by synthetic FAP-A-(269–292) peptides containing Ala substitutions. Control peptide is a random sequence of amino acids in FAP-A-(269–292). A, one representative experiment showing actual *M. bovis* BCG attachment. Each point is the mean ± S.D. of triplicate wells. B, percent inhibition was calculated for three individual experiments and reported as mean percent inhibition. Student’s *t* test showed the *p* values for Ala$^{273}$, Ala$^{274}$, Ala$^{275}$, and Ala$^{276}$ to equal 0.015, 0.015, 0.013, and 0.022, respectively.
A276A) showed partial abrogation of the ability of each peptide to inhibit M. bovis BCG attachment to fibronectin. When the four essential amino acids (RWFV) were either all changed to Ala (FAP-A4ala-(255–258)) or deleted (FAP-A4d-(255–258)), no functional activity of the respective peptides was observed (Fig. 4, B and C). The control mutants in which the four amino acids outside the fibronectin binding region were substituted with Ala (NGQI, amino acids 255–258, FAP-A4ala-(255–258)) or deleted (FAP-A4d-(255–258)) retained functional activity. These data indicate that one Ala substitution within the RWFV motif is not sufficient to completely abrogate fibronectin binding function in the recombinant FAP-A protein; however, the en bloc deletion of RWV or Ala substitution of the RWFV motif resulted in complete loss of fibronectin binding activity. These data demonstrate RWFV to be critical to the fibronectin binding function of mycobacterial FAP.

**DISCUSSION**

We performed studies to identify the amino acids necessary for fibronectin binding of FAP. Because previous studies demonstrated the FAP-A-(259–292) fibronectin binding peptide to be sufficient to block mycobacterial interaction with fibronectin, this site in FAP-A was characterized. The minimal binding sequence of FAP-A for fibronectin, consisting of 12 amino acids (amino acids 269–280), was identified. Using this binding sequence as a base, a panel of synthetic peptides containing single Ala substitutions was used to determine which amino acids were necessary for fibronectin binding (Fig. 2, B and C). Characterization of the functional effects of single Ala substitutions in the synthetic peptides showed that substitution at amino acids 273–276 showed partial inhibition of M. bovis BCG binding (Fig. 3, A and B). The amino acids RWFV are 100% conserved in all FAP molecules identified to date (Fig. 2D). These data show that the RWFV motif is essential for peptide binding to fibronectin and also is important in the M. bovis BCG/fibronectin interaction.

To validate the synthetic peptide data, additional studies were performed in which mutant recombinant proteins containing Ala substitutions or amino acid deletions were tested for fibronectin binding function. In these studies the ability of mutated recombinant FAP-A-(210–381) to inhibit M. bovis BCG binding to FN was compared with wild type FAP-A-(210–381). FAP-A mutants with single Ala substitutions at amino acids 273–276 showed partial inhibition of M. bovis BCG attachment to fibronectin. The Ala mutation of Arg at position 273 in recombinant FAP-(210–381) did not result in complete abrogation of function as was seen in the synthetic peptide. This suggests that the function of Arg273 was exaggerated in the shorter synthetic peptide.

FAP-A mutants in which all four relevant amino acids (273–276) were simultaneously mutated to Ala or were simultaneously deleted showed complete loss of function. Control proteins in which Ala deletion or substitution was performed at a site outside the fibronectin binding region (amino acids 255–258, NGQI) retained function. Taken together, these data demonstrate that the RWFV sequence is a critical functional motif in FAP binding to fibronectin. Moreover, because the RWFV motif was shown to be important in modulating M. bovis BCG attachment to fibronectin, the data suggest an important role for the motif in the M. bovis BCG/fibronectin interaction.
been implicated in the fibronectin binding of mycobacteria. Abou-Zeid et al. (8) first reported the interaction between antigen 85 complex proteins and fibronectin using culture supernatants of mycobacteria. The participation of the antigen 85 complex was established by probing Western blots of recombinant 85A, -B, and -C proteins to fibronectin (10–12, 15). A highly conserved motif among the antigen 85 complex proteins, FEWYYQ, was identified as an important sequence in the binding of recombinant 85 complex proteins to fibronectin (12). While the interaction between antigen 85 complex proteins and fibronectin has been demonstrated in vitro binding assays, the role of these proteins in the attachment of viable bacteria to fibronectin has not been established.

The relationship between the antigen 85 complex and FAP in the mycobacterial interaction with fibronectin is not known. The minimal amino acid sequence that will bind fibronectin was identified for the 85B protein and consists of 11 amino acids (FEWYYQSGLSV) (12). The amino acid composition of this binding peptide consists of 8 polar and 3 non-polar amino acids with a neutral net charge. Essential amino acids in the minimal binding sequence were identified as FEWYYQ, which has a net negative charge. FAP contains no region of homology with the identified 85B binding region. Furthermore, the composition of the minimal amino acid sequence of the FAP fibronectin binding region, which consists of 12 amino acids, is quite distinct from that of the 85B sequence. The FAP minimal binding peptide consists of 7 polar and 5 non-polar amino acids with a net positive charge. Moreover, the essential binding motif of this binding region, RWFV, bears little resemblance to the 85B sequence, FEWYYQ. The FAP sequence is highly non-polar and is positively charged while the 85B sequence is negatively charged and is comprised of 2 non-polar and 3 polar amino acids. The composition of the respective binding sites for antigen 85B and FAP suggest distinct function. In this regard, a previous report localized the binding site on fibronectin for 85B to be in the collagen binding region at the amino-terminal end of fibronectin (11). Consistent with this observation, the interaction between 85B and fibronectin was inhibited by gelatin. In contrast, our unpublished studies show that both FAP and intact M. bovis BCG attach to the carboxyl-terminal region of fibronectin containing the heparin binding site. In collaborative studies with Dr. James McCarthy (University of Minnesota), we have shown M. bovis BCG and FAP to bind to the 33/66 fragment of a trypsin/cathepsin D digest of fibronectin. In addition, previous studies showed M. bovis BCG attachment to fibronectin to be inhibited by heparin but not gelatin (2).

Comparison of FAP and protein 85B with other bacterial fibronectin attachment proteins suggests that FAP exhibits unique properties, whereas protein 85B shares many characteristics with these proteins. The best characterized of the fibronectin-binding proteins is that of Staphylococcus. The primary fibronectin binding sequence of Staphylococcus aureus is located in the 37–48 amino acid sequence that is repeated four times in the binding protein (16). The essential binding motif is a negatively charged hydrophilic motif, DFEEDT, and is shared by other Gram-positive cocci for which fibronectin attachment proteins have been identified (17). The negative charge and the hydrophilic nature of the Staphylococcus fibronectin binding protein is similar to the characteristics of the identified fibronectin binding sequence for the mycobacterial 85B protein. In addition, the region in the fibronectin molecule to which the Staphylococcus protein binds is located in the same collagen binding region as 85B (11, 16).

The studies we report here identify and characterize a fibronectin binding region of FAP-A. The data show that FAP-A binding depends on the RWFV sequence, which possesses characteristics distinct from antigen 85B and fibronectin-binding proteins of Gram-positive cocci. Further studies are needed to characterize the respective roles of FAP and the antigen 85 complex in mycobacterial attachment to fibronectin.

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