The Novel Fibronec tin-binding Motif and Key Residues of Mycobacteria*

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The binding motifs of the immunodominant antigen (Ag) α-Ag (Ag 85 complex B) of Mycobacterium kansasi for human fibronec tin were examined using digested fragments. We defined two fibronec tin-binding epitopes on 27 amino acids from 84 to 110 and on 20 amino acids from 211 to 230. The epitopes were almost conserved in the closely related Ag 85 complex of other mycobacteria species. Inhibition of fibronec tin binding to intact α-Ag molecules was observed with peptide-(84–110), but not with peptide-(211–230). Peptide-(84–110) could also inhibit fibronec tin binding to all components of the Ag 85 complex of Bacillus Calmette-Guérin (Ag 85A, Ag 85B, and Ag 85C). Further study with synthetic peptides defined 11 residues from 98 to 108 as the minimum motif. Six residues (98–FEWYYQ103) were critical for interacting with fibronec tin. The motif revealed no homology to other known prokaryotic and eukaryotic fibronec tin-binding proteins. The defined motif of α-Ag is novel and unique for mycobacteria.

In recent years, there has been a dramatic increase in mycobacterial disease even in some developed countries (1). The incidence has been associated with an increase in the number of individuals infected with human immunodeficiency virus-1 or patients whose immune systems have been compromised by immunosuppressive agents used to treat other diseases (2). Mycobacterium tuberculosis, Mycobacterium avium-intracellulare complex and Mycobacterium kansasi are the most frequently isolated mycobacteria from AIDS patients (3), and disseminated infection due to M. kansasi is a well established feature of immunocompromised patients (4, 5). Furthermore, M. kansasi has been implicated in pulmonary disease and has been reported as a cause of cutaneous infections and osteomyelitis (6, 7).

The elucidation of the mechanism of these infections and interactions with host immune systems is needed. We have been particularly interested in proteins secreted by mycobacteria. These proteins may play important roles not only in the establishment, progress, and continuation of the infection, but also in the host defense system since live mycobacteria can provoke protective immunity against tuberculosis, but killed organisms cannot (8). α-Antigen (Ag), 1 also known as Ag 85B (9), Ag 6 (10), and MPT59 (11), is one of the most dominant secretory proteins (12) and is a major stimulant of cellular and humoral immunity (11, 13–15). It is widely distributed among M. tuberculosis, Bacillus Calmette-Guérin (BCG) isolated from Mycobacterium bovis, and atypical mycobacteria (16). This Ag belongs to the Ag 85 complex, which consists of three structurally related components, Ag 85A, Ag 85B (α-Ag), and Ag 85C.

In the immune response, the Ag motif is characterized by the ability to bind to human fibronec tin (FN) (9) and has recently been defined as a mycolyltransferase, which is an important enzyme for unique mycobacterial cell wall synthesis (17). α-Ag induces interferon-γ (18, 19) and protective immunity against M. tuberculosis infection (20–22) and mediates attachment of whole bacteria to FN-coated surfaces (23–26). It has been suggested that binding to FN may represent the first step in the attachment and entry of mycobacteria into host cells. α-Ag has been regarded as an important molecule for BCG-mediated antitumor activity in the treatment of superficial bladder carcinoma (23). Interestingly, this Ag is a stimulus for human monocytes to induce tumor necrosis factor-α and this stimulatory effect may be mediated through plasma FN (24, 25). We have cloned and sequenced the genes encoding α-Ag of BCG (28), M. kansasi (29), M. avium (30), M. intracellulare (31), and Mycobacterium serofalaceum (32). As a continuation of this work, we attempted to delineate the specificity of the interaction of α-Ag and FN molecules. The novel motif required for FN binding and the contribution of the individual residues were investigated.

EXPERIMENTAL PROCEDURES

Bacterial Strain—BCG strain Tokyo was used in this study. It was grown at 37 °C in Sauton medium (33). Transformed BCG was maintained with kanamycin (20 μg/ml).

Media and Reagents—Peroxidase-conjugated swine anti-rabbit immunoglobulins was purchased from Dako A/S Co. (Glostrup, Denmark). FN was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Trypsin, which was modified by reductive alkylation and treated with tosylphenylalanyl chloromethyl ketone, was purchased from Promega (Madison, WI). Reagents used for synthesis and analysis were reagent-grade. Amino acid derivatives were purchased from Watanabe Chemical (Hiroshima, Japan).

Antigens—M. kansasi α-Ag was purified from culture filtrate (CF) of transformed BCG harboring plasmid pJK-1, which contains the gene encoding M. kansasi α-Ag (34). BCG α-Ag (Ag 85B), Ag 85A, Ag 85C, and MPB51 (35) were purified from CF derived from BCG. The procedure to purify antigens was developed on the basis of the purification technique for MPT59 as described previously (36).

Antibodies—Rabbit antibodies raised against FN were prepared as described previously (24).

Digestion of M. kansasi α-Ag—Purified M. kansasi α-Ag (1 mg/ml) was digested by overnight incubation at room temperature with CNBr (10 mg/ml) in 70% formic acid. The reaction mixture was dried under a stream of N2. M. kansasi α-Ag (125 μg/ml) was also digested with trypsin (5 μg/ml) at 37°C for 36 h. The digested mixture was precipitated with (meth)ethyl(methyl)glycine; BSA, bovine serum albumin; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay.
tated with 10% (w/v) trichloroacetic acid. These digests were stored at -20 °C.

**Binding of FN to CF Proteins and Digested Fragments of M. kansasii α-Ag**—The CF proteins (40 μg) were separated by two-dimensional gel electrophoresis as described previously (37). The digested fragments were separated by Tricine (N-tris(hydroxymethyl)methyl glycine)/SDS-polyacrylamide gel electrophoresis (38) and two-dimensional gel electrophoresis. Gels were stained with Coomassie Brilliant Blue or transblotted onto Immobilon membranes (Millipore Corp., Bedford, MA). The membranes were blocked with 3% bovine serum albumin in phosphate-buffered saline (BSA/PBS) for 1 h at 37 °C and probed with FN at 10 μg/ml in BSA/PBS for 1 h at 37 °C. Membranes were washed with 0.05% Tween 20 in PBS at 37 °C, and bound FN was detected with anti-FN antibodies followed by peroxidase-conjugated swine anti-rabbit immunoglobulins. Enzyme activity was visualized with 3,3’-diaminobenzidine and hydrogen peroxide in 0.05 M Tris-HCl, pH 7.5.

**Protein Sequencing**—The blotted membranes were stained with Coomassie Brilliant Blue. The stained bands and spots were cut out and applied to an Applied Biosystems 477A gas-phase protein sequencer (Applied Biosystems, Foster, CA). Then, the sequence of the five N-terminal amino acid residues of each sample was determined.

**Synthesis of Peptides**—The peptides were synthesized using standard Fmoc (N-(9-fluorenylethoxycarbonyl) chemistry (39). The synthesized peptides were purified by high pressure liquid chromatography with a RESOURCE RPC reversed-phase column (6.4 × 30 mm, 1 ml; Pharmacia Fine Chemicals, Uppsala, Sweden). Elution was carried out with a linear gradient of 16–32% acetonitrile in 0.1% NH₄HCO₃, pH 8.3, for 20 min and was monitored at 220 nm. For purification of only peptide-(211–230), 0.1% trifluoroacetic acid was used as buffer. The final products were identified by amino acid analysis. The peptides were lyophilized, and the concentrations were defined by weight.

**Enzyme-linked Immunosorbent Assay (ELISA) for FN Binding**—The FN binding to proteins was examined by solid-phase ELISA. SUMILON 96-well ELISA plates were coated with 10 μg/ml proteins/well in carbonate buffer, pH 9.6. Nonspecific sites were blocked by incubation with BSA/PBS. After washing, the plates were incubated with 2 μg FN/ml in BSA/PBS for 1 h at 37 °C. Bound FN was detected by rabbit anti-FN antibodies followed by peroxidase-conjugated swine anti-rabbit immunoglobulins and was subsequently developed with o-phenylenediamine dihydrochloride. The activity of synthetic peptides to bind to FN was determined as follows. SUMILON 96-well ELISA plates were coated with 100 μl of 6.28 μM peptides in carbonate buffer, pH 9.6, for 24 h at 37 °C. After blocking nonspecific sites with BSA/PBS, the quantity of FN (2 μg/ml) in BSA/PBS bound after 1 h of incubation at 37 °C was assayed as described above.

**Peptide Inhibition Assay: FN and α-Ag**—The capacity of synthetic peptides to interfere with protein binding to FN was examined. SUMILON 96-well ELISA plates were coated for 2 h at 37 °C with 0.5 μg proteins/well in carbonate buffer, pH 9.6. Nonspecific sites were blocked by incubation with BSA/PBS. After washing, the plates were incubated with 2 μg of FN/well in BSA/PBS for 1 h at 37 °C. Bound FN was determined by rabbit anti-FN antibodies followed by peroxidase-conjugated swine anti-rabbit immunoglobulins, and was subsequently developed with o-phenylenediamine dihydrochloride. The activity of synthetic peptides to bind to FN was determined as follows. SUMILON 96-well ELISA plates were coated with 10 μg of Ag 85 complexes (lanes 1 and 2) and blotted onto Immobilon membranes. The membranes were probed with FN (B, C lanes 3 and 4, and E), C lanes 1 and 3, CNBr-digested M. kansasii α-Ag; lanes 2 and 4, trypsin-digested M. kansasii α-Ag. The arrows indicate trypsin-1 spots A and B. The relative molecular masses (in kilodaltons) are shown to the left of each panel. B-α, BCG α-Ag.

**RESULTS**

**FN Binding to CF Proteins**—Fig. 1A shows the two-dimensional gel electrophoresis of CF proteins derived from BCG. The labeled spots were identified as described previously (35). FN bound not only to the Ag 85 complex, but also to MPBS1. There were some additional spots around the Ag 85 complex. These spots might be due to streaking of the Ag 85 complex occurring in the first dimension.

**Mapping of the FN-binding Epitope on M. kansasii α-Ag**—Purified M. kansasii α-Ag was digested by CNBr or trypsin to investigate the FN-binding sites. The digested fragments were analyzed by Tricine/SDS-polyacrylamide gel electrophoresis. CNBr digestion generated three bands from M. kansasii α-Ag that had molecular masses of 10.5, 6.2, and 5.8 kDa (Table I, CNBr-1, CNBr-2, and CNBr-3). Trypsin digestion generated three bands that had molecular masses of 8.0, 6.1, and 5.7 kDa (Table I, trypsin-1, trypsin-2, and trypsin-3). The other fragments were too small to see on Tricine/SDS-polyacrylamide gel electrophoresis. FN bound to the CNBr-3 and trypsin-1 bands (Fig. 1C). In control experiments, M. kansasii α-Ag and the digested bands did not react with anti-FN antibodies. The sequence of the first five N-terminal residues of each digested band was determined. From the sequence data of the fragments, the sequence of the first five N-terminal residues of each digested band was determined. From the sequence data of the fragments, the sequence of the first five N-terminal residues of each digested band was determined.

**Fig. 1.** Binding of FN to CF proteins and digested M. kansasii α-Ag fragments. The CF proteins (40 μg) were separated by two-dimensional gel electrophoresis (A and B). The digested M. kansasii α-Ag fragments were separated by Tricine/SDS-polyacrylamide gel electrophoresis (C) or two-dimensional gel electrophoresis (D and E). The gels were stained with Coomassie Brilliant Blue (A, C lanes 1 and 2, and D) and blotted onto Immobilon membranes. The membranes were probed with FN (B, C lanes 3 and 4, and E), C lanes 1 and 3, CNBr-digested M. kansasii α-Ag; lanes 2 and 4, trypsin-digested M. kansasii α-Ag. The arrows indicate trypsin-1 spots A and B. The relative molecular masses (in kilodaltons) are shown to the left of each panel. B-α, BCG α-Ag.

| Band/slot | Apparent size | FN binding | Sequence | Supposed position | Expected size |
|-----------|---------------|-------------|-----------|-------------------|--------------|
| CNBr-1    | 10.4          | –           | WGPSS... | Trp^{220}-Met^{214} | 10.4         |
| CNBr-2    | 6.1           | –           | PGGQG... | Pro^{111}-Met^{167} | 6.1         |
| CNBr-3    | 5.8           | +           | GRSIK... | Gly^{327}-Met^{310} | 5.8         |
| Trypsin-1 | –             | + A         | AQDDY... | Ala^{154}-Arg^{153} | 7.9         |
| Spot A    | 8.0           | + SV       | PT...    | Ser^{154}-Arg^{250} | 7.8         |
| Spot B    | 7.9           | + SDVPP... | Ser^{204}-Lys^{215} | 6.1         |
| Trypsin-2 | 6.1           | –           | SSNL...  | Ser^{770}-Ala^{725} | 5.5         |
| Trypsin-3 | 5.7           | –           | –         | –                 | –            |
acids 216–219, was prepared to improve its binding efficiency against the ELISA plates. Both peptides bound FN significantly above background levels when coated on the ELISA plates (Fig. 3). In M. kansasii α-Ag, at least two distinct and discontinuous FN-binding epitopes were identified at amino acids 84–110 and 211–230.

Inhibition of FN Binding by Peptides—We tested whether the peptides could inhibit the binding of FN to the M. kansasii α-Ag molecule. Peptide-(84–110) inhibited the binding of FN to M. kansasii α-Ag (Fig. 4). On the other hand, peptide-(211–230) had no effect on the interaction of FN and M. kansasii α-Ag even with the peptide concentration raised to 150 μM (data not shown). Peptide-(84–110) could also inhibit the binding of FN to BCG α-Ag, Ag 85A, Ag 85C, and MPB51 (Fig. 4). Limiting FN-binding Motif—Using a series of systematically shortened lengths, a fine FN-binding motif was identified (Fig. 5). A significantly decreased inhibition of FN binding to M. kansasii α-Ag was associated with the removal of Phe98. Deletion of Val108 from the C terminus resulted in complete loss of inhibition. The critical residues required for FN binding were 11 residues (FEWYYQSGLSV) that corresponded to peptide-(98–108).

Analysis of the Motif by Single Residue-substituted Analogues of Peptide-(98–108)—A series of peptides containing a single substitution with alanine was prepared to identify the residues within amino acids 98–108 that were critical for binding to FN. Fig. 6 shows the inhibition of FN binding to M. kansasii α-Ag by these peptides. Substitutions at positions 98–103 dramatically reduced inhibition. The Glu99→Ala and Tyr102→Ala peptides reduced the inhibitory activity by 15–17%. The Phe106→Ala, Trp100→Ala, Tyr101→Ala, and Gln103→Ala peptides gave no inhibition. The Ser104→Ala, Leu106→Ala, Ser107→Ala, and Val108→Ala peptides reduced inhibition slightly. No marked difference was observed in the Gly105→Ala peptide.

DISCUSSION

Pathogenic bacteria attach to their preferred host target by molecules on the bacterial surface, termed adhesins, that recognize cognate host-cell receptors (40). α-Ag and its families have not been suggested to be one of the bacterial ligands that can achieve attachment to a bridging ligand of host origin extracellular matrix FN. This Ag is specific for mycobacteria, and its interaction with FN might also enable the pathogen to reach a natural pathway determined by the bridging ligand FN-host receptor interaction. We examined the interaction of α-Ag with FN to elucidate its role in mycobacterial infection.

At least two different FN-binding epitopes were defined in M. kansasii α-Ag, amino acids 84–110 and 211–230. There is...
no sequence similarity between defined epitopes. These epitopes have no homology to other known prokaryotic and eukaryotic FN-binding proteins except the Ag 85 complex. Therefore, the Ag 85 complex might have unique abilities to bind to FN.

In the peptide inhibition assay, only peptide-(84–110) could inhibit the binding of FN to intact *M. kansasii* α-Ag. Each peptide had no effect on the binding of FN to the other (data not shown). The relative positions of these two epitopes within the three-dimensional structure of *M. kansasii* α-Ag are still unknown, but the results indicate that the multiple epitopes of *M. kansasii* α-Ag may work separately for binding to FN. Amino acids 211–230 might be hidden in the intact natural molecule and not hidden in the denatured state. In contrast, amino acids 84–110 might be exposed on the surface of the molecule and work as a major domain to bind to FN.

The multiple FN-binding regions had been previously reported in α-Ag of *Mycobacterium leprae* (25) and *M. bovis* (41), but their role in the natural α-Ag molecule in FN binding had not yet been examined. In this study, we defined a new region (amino acids 84–110) that might play a very important role in FN binding to the α-Ag molecule. This region and its surrounding sequence are almost identical among mycobacterial species including the other structurally related components, Ag 85A and Ag 85C. Peptide-(84–110) could inhibit the binding of FN to all components of the Ag 85 complex of BCG. The defined region may contain the common motif of the Ag 85 complex for binding to FN.

We attempted successively to determine the common motif of α-Ag using a series of peptides that truncated at the N and C termini. The peptide inhibition assay was performed to exclude the artifacts that might result from a different efficiency in peptide fixation to the ELISA plates. Concerning deletion at Ala97, ELISA could not detect the binding of FN to the wells that were coated with the peptide (data not shown), but the same peptide showed the full binding inhibition of FN as an original peptide. Ala97 might work to interact with the ELISA plates, but not with FN. The results indicated that the binding
motif contained 11 residues, \(98^\text{FEWYYQSGLSV}^{108}\).

Further study defined critical amino acid residues in this motif using analogous peptides that were substituted with alanines. Substitution with alanine allowed essential residues motif using analogous peptides that were substituted with Tyr102 can change place with the negatively charged residues amino acid substitutions. The aromatic residues Trp100 and revealed that the defined FN-binding motif allowed some Comparing the amino acid sequences among these antigens essential for binding to FN. Peptide-(98–108) also could inhibit the motif contained 11 residues, \(98^\text{FEWYYQSGLSV}^{108}\).

A substitution Val 108 into Ala did not affect the inhibition ability. However, the deletion of this residue removed the binding ability completely. The residue at this position is absolutely required, although it can be replaced with other hydrophobic residues. This is the first report on the ability of MPB51 to bind to FN. The secreted protein MPB51 is one of the major proteins in the CF derived from BCG and immunologically cross-reacts with the Ag 85 complex. We have defined the complete sequence of this Ag. MPB51 showed 37–43% homology to the components of the Ag 85 complex (35). MPB51 could bind to FN. Peptide-(84–110) could inhibit the binding of FN to MPB51. Interestingly, there is no sequence similarity between the peptide and MPB51. The Ag 85 complex and MPB51 might share the same binding position on FN. It is very meaningful to analyze their roles in pathogenesis and host immunity.

The motif does not overlap with the region that corresponds to the monoclonal antibody HYT27 binding determinant, amino acids 111–119 (42). There is good agreement with previous studies that HYT27 failed to block \(\alpha\)-Ag binding to FN (23). Immunoglobulins that recognize the FN-binding motif may enhance mycobacterial binding to FN. The knowledge presented in this study might be useful in the control of mycobacterial infection. We propose the motif, particularly the key residues \(98^\text{FEWYY}^{108}\), as a possible candidate component of a subunit synthetic vaccine against mycobacterial infection.

Studies to examine the binding sites on FN molecules that interact with \(\alpha\)-Ag are currently underway in our laboratory. Our findings may contribute to clarification of the roles of \(\alpha\)-Ag in the mycobacteria-host cell relationship.

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