The domains of human fibronectin mediating the binding of α antigen, the most immunopotent antigen of mycobacteria that induces protective immunity against mycobacterial infection

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We have recently shown that α antigen (α -Ag), the immunodominant antigen of mycobacteria, has a novel fibronectin (FN)-binding motif that is unique among mycobacteria [Naito, Ohara, Matsumoto and Yamada (1998) J. Biol. Chem. 273, 2905–2909]. In this study, we examined the domains of human FN that interacted with α -Ag. Fragments of FN generated by either proteolysis or recombinant DNA techniques were compared for their ability to bind to α -Ag. Fragments containing either the C-terminal heparin-binding domain or the central cell-binding domain consistently bound to α -Ag. The fragment of the C-

terminal heparin-binding domain, upon mutation that resulted in the loss of its heparin-binding activity, could not bind with α -Ag. These findings suggested that the mutated site, i.e. the main heparin-binding site of FN, was also the principal site for binding to α -Ag. The α -Ag-binding domains of FN could bind whole mycobacterial bacilli, suggesting that these two domains are important contributors to mycobacterial infection.

Key words: α antigen, fibronectin, mycobacteria.

INTRODUCTION

Mycobacterial infection is a major health problem and a leading cause of world-wide morbidity and mortality, mainly because of the non-efficacy of existing vaccines and the increased prevalence of the disease in AIDS patients. Mycobacteria are intracellular pathogens that multiply within host cells. The mechanisms of this infection, and the subsequent immunopathogenesis, are not yet fully understood. The antigens of mycobacteria, including the secreted proteins, have been implicated in both the protective immune responses and the pathogenesis. Since only live mycobacteria can provoke protective immunity against tuberculosis [1], we have been particularly interested in the proteins secreted by mycobacteria.

α Antigen (α -Ag), also known as Ag 85B, is one of the most predominant secretory proteins of mycobacteria [2] and is widely distributed among *Mycobacterium tuberculosis*, Bacille Calmette–Guérin (BCG) and atypical mycobacteria [3]. We first cloned the gene for α -Ag from BCG [4], *M. kansasii* [5], *M. avium* [6], *M. intracellulare* [7] and *M. scrofulaceum* [8]. α -Ag is an enzyme that functions in mycolic acid synthesis [9], and is capable of inducing cellular immunity in the host [2,10,11]. α -Ag induces interferon- γ synthesis [12,13] and protective immunity against tuberculosis [14] and leprosy [15], and is suggested to be the main target for the humoral immunity of untreated patients [16]. This antigen was characterized as fibronectin (FN)-binding protein [17]. α -Ag also exists on bacterial surfaces, where it mediates the attachment of whole bacteria to FN-coated surfaces [18–21]. It has also been suggested to be a bacterial ligand that can attach itself to a bridging ligand of the host FN.

FN is a mammalian glycoprotein that is ubiquitous in body fluid, the extracellular matrix (ECM) and basement membranes [22]. FN is composed of repeating structural units, as determined from sequence homology studies, which are designated type I, type II and type III modules, and illustrated in Figure 1. These

modules are numbered from the N-terminus of the molecule, and consist of multiple functional domains. FN has specific binding sites for a number of proteins, including collagen, heparin and integrins. FN serves various biological functions, including roles in metastasis, wound healing, embryogenesis, blood clotting and phagocytosis. FN might also be involved in host cell activation and migration via its binding to integrins and cell-surface molecules.

Interestingly, α -Ag acts as a stimulus for human monocytes to induce tumour necrosis factor- α , and this stimulatory effect may be mediated via plasma FN [23]. In patients with active tuberculosis, α -Ag exists in the serum as a complex with FN [24]. The immunological significance of the interaction between α -Ag and FN in mycobacterial infection, however, remains unclear, and we therefore attempted to clarify the mechanism of this interaction. First, we defined a novel motif on α -Ag that binds to FN [25]. This motif revealed no homology with other known prokaryotic or eukaryotic FN-binding proteins. The defined motif on α -Ag is also unique among mycobacteria. A major future step of our work will be the characterization of the α -Ag-binding domains on the FN molecule. In the present paper, we present evidence that the α -Ag-binding sites on the FN molecule are located within the central cell-binding domain and the C-terminal heparin-binding domain.

EXPERIMENTAL

Materials

Human plasma FN and biotin were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Thermolysin, heparin–albumin–biotin and FITC were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Biotinamidocaproate *N*-hydroxysuccinimide ester (Biotin-X-NHS) was purchased from Calbiochem–Novabiochem (La Jolla, CA, U.S.A.). Peroxidase-conjugated goat anti-mouse immunoglobulins and

Abbreviations used: α -Ag, α antigen; BCG, Bacille Calmette–Guérin; FN, fibronectin; ECM, extracellular matrix; Biotin-X-NHS, biotinamidocaproate *N*-hydroxysuccinimide ester; GST, glutathione S-transferase; CBB, Coomassie Brilliant Blue; CCB, the central cell-binding domain of FN.

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Figure 1 Structure of FN, digested fragments of FN and bacterially expressed GST fusion fragments

Modular structures of FN are shown schematically on the basis of internally homologous modules termed types I, II and III. The IIICS segments are shown with a hatched oval. Functional domains that interact with heparin (Hep-1 and Hep-2), fibrin (Fib-1 and Fib-2), collagen and cell-surface integrins (CCBD) are labelled above the respective domains. Recombinant FN fragments encompassing different segments of type III modules were produced as fusion proteins with GST. The RGD cell-binding sequence (+) and the high-affinity heparin-binding sequence (*) are illustrated below the clear boxes. The mutated high-affinity heparin-binding sequence (Δ) is also shown, as described above. The thick lines indicate thermolysin-digested fragments. K, kDa.

peroxidase-conjugated swine anti-rabbit immunoglobulins were obtained from Dako A/S Co. (Glostrup, Denmark). Peroxidase-conjugated streptavidin was purchased from Chemicon Int. Inc. (Temecula, CA, U.S.A.). The reagents used for the synthesis and analysis were of reagent grade. α -Ag of *M. kansasii* was purified as described previously [26]. The biotinylation of α -Ag was performed with 46% (w/w) Biotin-X-NHS (3 mg/ml α -Ag, 1.4 mg/ml Biotin-X-NHS) in PBS, pH 7.4, at 25 °C for 16 h. The mixture was subsequently dialysed against PBS (changing the dialysis buffer several times) at 4 °C to remove excess biotin, concentrated and stored at -20 °C. The peptide 91–110, which corresponds to the FN-binding epitope of α -Ag, was synthesized and purified as described previously [25]. Rabbit antibodies raised against FN were prepared as described previously [19]. Mouse antibodies raised against glutathione S-transferase (GST) were prepared by immunizing purified GST with Freund's incomplete adjuvant (Difco Laboratories Inc., Detroit, MI, U.S.A.).

Preparation of recombinant proteins

The transfected CHO DG44 cell line that expressed the truncated form of FN, consisting of the N-terminal 70 kDa and C-terminal 37 kDa regions and referred to as 'mini FN' (Figure 1), was prepared as described previously [27]. The transfected cells were grown in α -minimum essential medium (Gibco BRL, Gaithersburg, MD, U.S.A.) containing 1% (v/v) FN-free fetal bovine serum that was prepared by passing serum through a gelatin-affinity column (Pharmacia Biotech, Uppsala, Sweden). The mini FN was purified from the cultured medium by affinity chromatography with a gelatin-affinity column. The recombinant fragments, GST-CH and GST-C (Figure 1), were prepared as GST fusion fragments, as described previously [28]. The cDNA

Table 1 Primers used in this study

Restriction sites for *Bam*HI or *Sal*I are shown in bold; the stop codon is shown in italics. The substituted nucleotides are underlined.

Primer	Sequence
III12F	5'-CGCGGATCCGCTATTCCTGCACCAACTGA-3'
III14R	5'-ACGGGTCGACCTA ATAGTCTACATCTCCCTGGGAATG TGACCAATTGGATTCTCTGTCTTTTCTCCCAATC-3'
Mutation primer	
Forward	5'-GCATCTGTACACGAGCGCTACTTGGTGGGCTGACATTC-3'
Reverse	5'-GAATGTACAGCCACCAAGTAGCGCTCGTGTGACAGATGC-3'

fragment encoding the III12–14 modules was amplified from pAIFNCT [28] by PCR using primers III12F and III14R (Table 1). These primers contained the sequences for *Bam*HI and *Sal*I restriction sites to facilitate ligation into the pGEX 4T-1 (Pharmacia Biotech). The plasmid was expressed in the *Escherichia coli* strain BL21, and the resulting GST fusion protein (designated GST-H; Figure 1) was purified on a glutathione-Sepharose column (Pharmacia Biotech), according to the manufacturer's instructions. The mutated form of the recombinant protein, with substitution of serine residues for arginine residues at the high-affinity heparin-binding site in the III13 module, was prepared by the method of Ho et al. [29]. cDNA fragments containing the desired mutations at 5' or 3' sequences were amplified from those encoding intact III12–14 modules by PCR using the mutation primers listed in Table 1, together with primers III12F or III14R. The resulting cDNA fragments containing mutation primers at either the 3' or 5' ends were mixed and annealed at their overlapping ends, followed by

a further round of amplification by PCR using the primers outside the III12–14 modules (i.e. III12F and III14R). The resulting cDNA containing mutations was ligated into pGEX 4T-1. The plasmid was expressed as described above; the resulting fusion protein was then designated GST-HP4. Since the replacement of arginine with serine on this site abrogated heparin-binding function [30], GST-HP4 corresponded to the non-functional Hep-2 (the C-terminal heparin-binding domain of FN, as opposed to Hep-1, which is the designation used subsequently for the N-terminal heparin-binding domain).

Binding of α -Ag to digested fragments of FN

Digestion of FN with thermolysin was performed as previously described [31], i.e. the experimental conditions were 0.5% (w/w) thermolysin (1 mg/ml FN, 5 μ g/ml thermolysin) in PBS/2.5 mM CaCl_2 , pH 7.2, for 4 h at 25 °C. The reaction was terminated by adding EDTA (final concentration 10 mM). The digested fragments were separated by SDS/PAGE, and the gels were stained with Coomassie Brilliant Blue (CBB) or transblotted on to Immobilon membranes (Millipore, Bedford, MA, U.S.A.). The membrane was blocked with 5% (w/v) BSA in PBS (BSA-PBS) for 1 h at 37 °C, and then probed with biotinylated α -Ag (10 μ g/ml) and 6.25 μ M biotin in BSA-PBS for 1 h at 37 °C. The membrane was washed with 0.05% (v/v) Tween 20/PBS. The bound biotinylated α -Ag and biotin were detected with peroxidase-conjugated streptavidin. Enzyme activity was detected with 3,3'-diaminobenzidine and hydrogen peroxide in 0.05 M Tris/HCl buffer, pH 7.5.

Protein sequencing

The blotted membrane was stained with CBB. The stained bands were cut out and applied to an Applied Biosystems 477A gas-phase protein sequencer (Applied Biosystems, Foster, CA, U.S.A.). The sequence of the N-terminal five amino acid residues of each sample was then determined.

ELISA for α -Ag binding assay

The binding of α -Ag to FN and mini FN was examined by solid-phase ELISA. SUMILON 96-well ELISA plates (Sumitomo Bakelite Co. Ltd., Tokyo, Japan) were coated with 100 μ l/well of 0.1 μ M FN or mini FN in PBS for 2 h at 37 °C. Non-specific sites were blocked by incubation with BSA-PBS. After washing, the plates were incubated with 2 μ g/well of biotinylated α -Ag and 0.2 μ g/well of heparin–albumin–biotin in BSA-PBS for 1 h at 37 °C. The bound biotinylated α -Ag and heparin–albumin–biotin were determined by peroxidase-conjugated streptavidin, and subsequently developed with *o*-phenylenediamine dihydrochloride.

ELISA for GST–FN fusion-fragments binding assay

The binding of GST–FN fusion fragments to α -Ag and peptide 91–110 was examined by solid-phase ELISA. The 96-well ELISA plates were coated with 0.5 μ g/well of α -Ag in PBS for 2 h at 37 °C, or 100 μ l/well of 6.25 μ M peptide 91–110 in 0.1 M sodium bicarbonate buffer, pH 9.6, for 24 h at 37 °C. Non-specific sites were blocked by incubation with BSA-PBS. After washing, the plates were incubated with 100 μ l of either 0.1 μ M GST–FN fusion fragments or GST in BSA-PBS for 1 h at 37 °C. The bound fragment was detected with mouse anti-GST antibodies and peroxidase-conjugated goat anti-mouse immunoglobulins, and subsequently developed with *o*-phenylenediamine dihydrochloride.

Inhibition ELISA

The ability of GST–FN fusion fragments to interfere with binding of α -Ag to intact FN was examined. The 96-well ELISA plates were coated for 2 h at 37 °C with 2 μ g/well of FN in PBS, pH 7.2. Then, non-specific sites were blocked by incubation with BSA-PBS, as described above. The biotinylated α -Ag in BSA-PBS (at a concentration of 0.66 μ M) were preincubated with GST–FN fusion fragments at 0.66 μ M and 6.6 μ M for 0.5 h at 37 °C. Subsequently, 100 μ l of the mixture was added to the wells. Bound α -Ag was detected with peroxidase-conjugated streptavidin, and subsequently developed with *o*-phenylenediamine dihydrochloride.

The ability of heparin and gelatin to interfere with binding of FN and GST–FN fusion fragments to α -Ag was examined. The 96-well ELISA plates were coated for 2 h at 37 °C with 0.5 μ g/well of α -Ag in PBS, pH 7.2. Then, non-specific sites were blocked as described above. The 0.1 μ M FN and GST–FN fusion fragments in BSA-PBS were preincubated with inhibitor at 0 to 100 μ g/ml for 1 h at 37 °C. Subsequently, 100 μ l of the mixture was added to the wells. Bound FN was determined by reacting with rabbit anti-FN antibodies and peroxidase-conjugated swine anti-rabbit immunoglobulins, and subsequently developed. The bound GST–FN fusion fragments were assayed as described above.

BCG binding assay

BCG substrain Tokyo was used in this study. It was grown to the early-exponential phase at 37 °C in Middlebrook 7H9 broth (Difco Laboratories Inc.) supplemented with 10% (v/v) albumin–dextrose–catalase enrichment (Difco Laboratories Inc.) and 0.5% (v/v) Tween 80. The cells were harvested by centrifugation, washed twice with 0.05% (v/v) Tween 80/PBS, and then labelled by incubation with 10 mg/ml FITC in 10% (v/v) DMSO/0.05% (v/v) Tween 80/PBS at 25 °C for 2 h. The labelled cells were then washed twice with 0.05% (v/v) Tween 80/PBS. The 96-well ELISA plates were coated for 2 h at 37 °C with 100 μ l of 0.5 μ M FN, GST or GST–FN fusion fragments, and non-specific sites were blocked with BSA-PBS. A total of 1.0×10^7 FITC-labelled BCGs in 100 μ l of 1% (w/v) BSA/0.05% (v/v) Tween 20/PBS were added to the appropriate wells. After incubation for 1 h at 37 °C, the wells were washed with 0.05% (v/v) Tween 20/PBS, and bound FITC-labelled BCGs were detected using a Fluoroskan II (Labsystems, Helsinki, Finland).

Sequence data analysis

The ScanProsite program in the ExPASy molecular server of SIB (Genève, Switzerland) was used to scan the amino acid sequence motif against the SWISS-PROT database. The FASTA program in DDBJ (Shizuoka, Japan) was used to search for amino acid sequence homologies in the DDBJ, PIR and SWISS-PROT databases.

RESULTS

Detection of digested fragments and mini FN, with ability to bind to biotinylated α -Ag

The limited digestion of FN with thermolysin produced 105 kDa, 67 kDa, 55 kDa, 50 kDa, 43 kDa, 34 kDa, 30 kDa, 28 kDa, 24 kDa and 22 kDa fragments, of which the 67 kDa and 55 kDa fragments thus generated reacted with biotinylated α -Ag and biotin. It is possible, however, that these fragments might react with biotin, and not α -Ag. α -Ag specifically bound to thermolysin-digested 105 kDa, 50 kDa, 34 kDa and 30 kDa

Figure 2 Binding of α -Ag to digested FN fragments

The digested FN fragments (2 μ g/lane) were separated with SDS/PAGE. Gels were stained with CBB (lane 1) and blotted on to Immobilon membranes (lanes 2 and 3). The membranes were probed with biotinylated α -Ag (lane 2) or biotin (lane 3). The molecular-mass makers (in kDa) are shown on the left; arrows on the right indicate the position of the α -Ag-bound fragments.

Figure 3 Binding of α -Ag and heparin to wells coated with FN and mini FN

The amounts of bound biotinylated α -Ag and heparin-albumin-biotin were determined by probing with peroxidase-conjugated streptavidin. Hatched bars and black bars represent binding of heparin-albumin-biotin and α -Ag respectively to the proteins described in the key. Each bar represents the mean $\pm$ S.D. for four independent measurements (wells).

fragments (Figure 2). According to the reported pattern of separation of digested FN fragments on SDS/PAGE [31,32], the thermolysin-digested 105 kDa fragment corresponded to the cell-binding domain (the III2–11 modules). The amino acid residues identified by N-terminal sequence analysis of thermolysin-digested 34 kDa and 30 kDa fragments (IIPAVP and AVT.IPAPT) were identical with the N-terminal sequence of the III8 and the III12 modules of FN respectively. Considering these data, and the apparent sizes of the fragments, the thermolysin-digested 34 kDa and 30 kDa fragments therefore corresponded to the central cell-binding domain (CCBD) (the III8–10 modules) and the C-terminal heparin-binding domain, i.e. Hep-2 (the III12–14 modules), respectively. We were unable to determine the N-terminal sequence of the thermolysin-digested 50 kDa fragment, and therefore could not identify its position. The digested fragments described above did not completely

Figure 4 Binding of GST-FN fusion fragments to wells coated with α -Ag and BSA

Bound GST-FN fusion fragments were detected with anti-GST antibodies. Each bar represents the mean $\pm$ S.D. for three independent measurements (wells).

Figure 5 Inhibition of α -Ag binding to wells coated with FN by GST-FN fusion fragments

Black bars and hatched bars describe α -Ag binding to FN with 0.66 μ M and 6.6 μ M proteins respectively. Each bar represents the mean $\pm$ S.D. for four independent experiments (wells).

overlap other domains of FN, such as the N-terminal heparin-binding domain (Hep-1/Fib-1) or the collagen-binding domain. Subsequently, we examined mini FN, which included these domains. α -Ag did not bind to solid-phase mini FN (Figure 3). The same result was also obtained from a separate experiment performed with solid-phase α -Ag and mini FN (results not shown). Thus the suggested target domains for α -Ag binding are CCBD and Hep-2. These results are integrated into the schematic representation of the domains of FN shown in Figure 1.

Ability of recombinant GST fusion fragments to bind to α -Ag

To define the binding domain in more detail, we prepared recombinant FN fragments, as illustrated in Figure 1, and examined their ability to bind to α -Ag. A significant binding of the GST fusion FN fragments GST-C, GST-H and GST-CH was observed, as shown in Figure 4. At least two distinct

Figure 6 Inhibition of FN and GST-FN fusion fragments binding to wells coated with α -Ag by heparin and gelatin

(A) Comparison of the abilities of heparin and gelatin to inhibit the binding of FN to wells coated with α -Ag. Bound FN was detected by ELISA with polyclonal anti-FN antibodies. (B) Comparison of the ability of heparin to inhibit the binding of GST-C and GST-H fragments to wells coated with α -Ag. Bound fragments were detected by ELISA with polyclonal anti-GST antibodies. Each point is the mean for four independent experiments. Statistical significance for the inhibition was determined by Student's *t* test analysis (*, $P < 0.01$; *, $P < 0.05$).

Figure 7 Binding of the mutated fragment, GST-HP4, to α -Ag and the peptide 91-110

GST, GST-C and GST-HP4 were added to wells coated with α -Ag (black bars) and BSA (white bars) (left) or peptide 91-110 (black bars) and BSA (white bars) (right). Bound fragments were detected as described in the legend to Figure 3. Each bar represents the mean $\pm$ S.D. for triplicate wells.

domains of FN, namely CCBD and Hep-2, were defined as the binding domains. The differences in the ability to bind α -Ag among GST-C, GST-H and GST-CH were found to be statistically insignificant, suggesting that two domains of GST-CH (CCBD and Hep-2) might compete with each other to bind α -Ag, rather than operating synergistically or co-operatively.

Competition assay

To evaluate the hypothesis above, the soluble recombinant FN fragments GST-H and GST-C were tested for their ability to influence the binding of α -Ag to whole FN. Both of the fragments inhibited the efficient binding of α -Ag (Figure 5). At a concentration of 6.6 μ M of these fragments, the binding was completely inhibited, suggesting that either of the α -Ag-binding domains could inhibit the binding of the other to α -Ag. Therefore Hep-2 and CCBD competed with each other for binding to α -Ag.

In order to characterize the binding further, the competitive inhibition of FN binding to α -Ag by heparin and gelatin was tested. Heparin inhibited the binding to an appreciable extent in a dose-dependent manner, whereas gelatin did not, as exemplified in Figure 6(A). At a concentration of 100 μ g/ml heparin, the percentage inhibition achieved was approximately 70%. Heparin inhibited the binding of the GST-H fragment to α -Ag more efficiently than that of the whole FN molecule (Figure 6B). Moreover, α -Ag seemed to share either a similar or an identical binding site with heparin on the Hep-2 domain. Heparin did not inhibit the binding of the GST-C fragment to α -Ag, suggesting that α -Ag has two distinct mechanisms for binding to FN.

Mutational analysis

The high-affinity heparin-binding site, Ser-Pro-Pro-Arg-Arg-Ala-Arg-Val, was characterized previously in the III13 module of

Figure 8 BCG binding to recombinant FN fragments

Wells were coated with the fragments indicated along the x-axis, and FITC-labelled BCG was then added. Each bar represents the mean $\pm$ S.D. for six wells.

Hep-2 [33]. To examine whether α -Ag could interact with this site, a mutant fragment GST-HP4, for which the heparin-binding activity had been lost, was prepared by amino acid substitution (see Figure 1 for details). GST-HP4 demonstrated no binding activity to either α -Ag or peptide 91–110, as shown in Figure 7. It was thus concluded that α -Ag might utilize the high-affinity heparin-binding site in the III13 module of the Hep-2 domain for binding to FN.

BCG binding to FN fragments

The recombinant FN fragments were examined to determine whether they play a role in the binding of whole mycobacteria. As shown in Figure 8, GST fusion FN fragments GST-C, GST-H, GST-CH and GST-HP4 were able to bind significantly to BCG; therefore Hep-2 and CCBD may contribute to the binding of BCG. Binding of BCG to GST-HP4, however, was lower than that for GST-H (Student's *t* test, $P < 0.01$), suggesting that the high-affinity heparin-binding site on Hep-2 may have a definable role in the binding of mycobacteria.

DISCUSSION

α -Ag is reported to be an immunodominant antigen and a FN-binding protein. We have recently determined the novel FN-binding motif of this antigen. As this motif possessed no homology with other known prokaryotic and eukaryotic FN-binding proteins, α -Ag was expected to show different binding to FN. This study was undertaken to determine the α -Ag-binding site of FN. We used both purified human FN and recombinant fragments of FN that were expressed in *E. coli*. The results obtained have shown that there is no difference in the binding capabilities of α -Ag with either system, suggesting that glycosylation of FN is not relevant for the binding of α -Ag. We have shown that α -Ag was able to bind to two distinct domains of FN, the C-terminal heparin-binding domain (Hep-2) and the central cell-binding domain (CCBD). The recombinant fragments corresponded to those domains that also bound to mycobacteria.

Pathogenic bacteria generally express surface proteins that possess an affinity for components of the mammalian ECM, and thereby facilitate the first steps of infection. FN is one of the

major components of ECM, existing in abundance in body fluid. Since FN can bind to other ECMs, FN, when bound, might lead to bacteria binding to other ECMs. The domains of FN to which various pathogenic bacteria bind have been characterized, and streptococci and staphylococci commonly bind to the N-terminal heparin-binding domain of FN, i.e. Hep-1, via the FN-binding repeats of bacterial surface proteins [34]. Many enterobacterial fimbriae were shown to bind to Hep-1, Fib-2 and the gelatin-binding domain of FN. The present study has shown that mycobacteria, in contrast, bind to different domains of FN, Hep-2 and CCBD, via α -Ag. These domains are rarely utilized as microbial targets. Only a few bacteria, such as *Streptococcus pneumoniae*, have been reported to possess the ability to bind to Hep-1 and Hep-2 [35]. This result was consistent with our expectations, and suggested a unique interaction between mycobacteria and FN.

Our study cannot completely exclude the possibility that α -Ag-binding sites might be lost following proteolytic digestion. However, the inhibition assay provided us with an indication of whether this might hold true. The recombinant fragments of α -Ag-binding domains, Hep-2 and CCBD, completely inhibited the binding of α -Ag to intact FN (Figure 5), and thus α -Ag might bind predominantly to Hep-2 and CCBD of whole FN. We anticipated that it was only a remote possibility that α -Ag possessed other binding sites on the FN molecule. Moreover, Hep-2 and CCBD competed with each other for binding to α -Ag, suggesting that these two domains might recognize either the same, or overlapping, sites of the α -Ag molecule with similar affinity.

Peake et al. have previously investigated the binding of FN to α -Ag, and obtained a different result, i.e. that α -Ag bound to the collagen-binding domain of FN [36]. This discrepancy might have resulted from the different preparation techniques used for α -Ag. We purified α -Ag as the intact native molecule without procedures involving denaturation or reduction, whereas Peake et al. utilized recombinant α -Ag. Therefore our results elucidated the interaction of the native α -Ag molecule to FN, consistent with previously performed studies. The observation that the interaction between α -Ag and FN was inhibited by heparin, but not by gelatin, was consistent with previous studies in which BCG attachment to FN was only inhibited by heparin, and not by gelatin [37]. Furthermore, the site-directed mutagenesis study revealed that α -Ag shared the same binding site with heparin. α -Ag bound to the high-affinity heparin-binding site in Hep-2 (sequence of PRRARV). This site consists of both hydrophobic and positively charged amino acid residues, the molecular characteristics of which could facilitate interaction with the negatively charged sulphate groups of heparin [33]. Interestingly, the FN-binding motif of α -Ag of sequence FEWYYQSGLSV consists of hydrophobic and negatively charged amino acid residues [25]. Thus the mechanism of the binding between α -Ag and Hep-2 might be of an ionic and hydrophobic nature, which is similar to heparin binding.

A different mechanism might be used for the interaction of CCBD with α -Ag. The peptide 91–110, which contained the FN-binding motif of α -Ag, recognized not only Hep-2, but also CCBD. Since this peptide could inhibit the binding of both CCBD and Hep-2 fragments to α -Ag (results not shown), α -Ag bound to two domains of FN via the same binding motif on its molecule. These data suggest that the FN-binding motif of α -Ag might have two distinct mechanisms to mediate binding with FN. Further conformational studies are needed to determine the detailed mechanism of the interaction between CCBD and α -Ag.

FN can control cell activation and migration via its binding to integrins and cell-surface molecules. The 'trigger' of these

processes is provided by the binding of integrins. This study could not delineate the detailed mechanism of the interaction between CCBD and α -Ag. CCBD contains the RGD motif (Figure 1) and other sites that are recognized by many integrins [28]. The RGD peptide could not inhibit the FN binding of with α -Ag (results not shown). α -Ag might not directly interact with the RGD motif on CCBD. However, the possibility remains that α -Ag interacts with other integrin-binding sites of CCBD, thereby altering the conformation of this domain. α -Ag bound to CCBD might therefore influence the cell–FN interaction. In fact, monocyte agglutination activity of human T-cell FN has been shown to be significantly inhibited by α -Ag *in vitro* [38]. It was reported that the production of α -Ag is increased at tissue sites where mycobacteria grow [39]. α -Ag might therefore be expected to modify the local effective response of host lymphocytes against infected mycobacteria. It might be important not only for the establishment, but also for the progress, of infection.

Although α -Ag is a predominantly secreted protein, some of the antigen is retained on the surface of mycobacteria [21]. Such surface-bound α -Ag might be utilized for mycobacterial attachment to FN. It might also conveniently play an enzymic role in cell-wall synthesis [9]. The mechanism whereby α -Ag may anchor to the mycobacterial surface has yet to be fully clarified. Unexpectedly, we have identified a possible factor contributing towards this mechanism: the PRRARV sequence of Hep-2, i.e. the α -Ag-binding site, is also found in the hypothetical 69.2 kDa acyltransferase of *M. tuberculosis* [40]. This mycobacterial acyltransferase is highly homologous with other known acyltransferases, as determined from an amino acid sequence homology search. Acyltransferase catalyses the biosynthesis of phospholipids, and, in *E. coli*, it exists as a membrane-integrated enzyme [41]. In mycobacteria, this enzyme may be expected to be expressed in the membrane. Therefore we can surmise that mycobacterial acyltransferase might help α -Ag to remain attached on the surface via its PRRARV sequence. Further studies are needed to elucidate this possibility, which could lead to a greater insight into the features of α -Ag in the physiology and the pathogenesis of mycobacteria.

The biological significance of the unique interaction between α -Ag and FN remains to be studied further. These studies might eventually lead to the efficient prevention of mycobacterial disease.

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