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# A *Mycobacterium leprae* Gene Encoding a Fibronectin Binding Protein Is Used for Efficient Invasion of Epithelial Cells and Schwann Cells

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***Mycobacterium leprae*, the causative agent of leprosy, is an obligate intracellular pathogen. *M. leprae* can infect a variety of cells in vivo, including epithelial cells, muscle cells, and Schwann cells, in addition to macrophages. The ligand-receptor interactions important in the attachment and ingestion of *M. leprae* by these nonmacrophage cells remains unknown. Fibronectin (FN) significantly enhances both attachment and ingestion of *M. leprae* by epithelial and Schwann cell lines. We cloned an *M. leprae* FN binding protein (FN attachment protein [FAP]) distinct from the 85ABC complex which has been shown previously to bind FN. The FAP open reading frame predicts a protein of 29.5 kDa with a 39-amino-acid signal peptide and was previously described as an antigen in leprosy patients. *M. leprae* FAP has homologies in *M. vaccae*, *M. avium*, and *M. tuberculosis*, as determined by Southern blotting and direct peptide analysis. Both anti-FAP antibodies and an *Escherichia coli*-expressed recombinant protein significantly blocked *M. leprae* attachment and internalization by T-24, an epithelial cell line, and JS1, a Schwann cell line. These data suggest that FN can be a bridging opsonic ligand for attachment of mycobacteria to nonphagocytes and that FAP plays an important role in this process. This may be an important step in the initiation of *M. leprae* infection in vivo.**

*Mycobacterium leprae* is an obligate intracellular pathogen and the causative agent of leprosy. Unlike other mycobacteria, which survive exclusively in macrophages in vivo, *M. leprae* resides in a number of cell types, including muscle cells, epithelial cells, and Schwann cells (7, 16, 17). Invasion and destruction of these nonmacrophage cell types by *M. leprae* is responsible for many of the clinical manifestations of leprosy. Furthermore, these host cells do not express complement or mannose receptors, which are important in infection of macrophages by mycobacteria (21). Little is understood about the ligand-receptor interactions important for invasion of nonmacrophages by *M. leprae.*

Binding to the extracellular matrix protein fibronectin (FN) is a virulence factor for several pyogenic bacteria, presumably because this property allows initial colonization of mucosal surfaces (9, 12). However, these bacteria are extracellular pathogens and there is no known role for FN in establishment of infection by obligate intracellular pathogens. In previous studies, we demonstrated that the ability to bind FN is a highly conserved property among mycobacterial species (2). *M. bovis* BCG binding to human bladder carcinoma cell line T-24 was shown to require FN, and the internalization of BCG by T-24 cells was mediated in part by recognition of BCG-bound FN by the $\alpha_5\beta_1$ integrin FN receptor expressed on the T-24 cells (14). While the pathogenic significance of infection of cells other than macrophages is unclear for most mycobacteria, it is a key element in the pathogenesis of *M. leprae* infection.

Several mycobacterial FN binding proteins have been identified (13). We previously reported the purification of a 50-kDa FN binding protein from *M. vaccae* culture supernatant (19). Monoclonal and polyclonal antibodies to the 50-kDa FN binding protein recognize a cell wall component in BCG (19) and several other mycobacterial species, including *M. tuberculosis*, *M. kansasii*, and *M. avium*. Antibodies to this protein inhibit the binding of several mycobacteria to FN, and in particular, both monoclonal and polyclonal antibodies to this FN attachment protein (FAP) inhibit BCG binding to T-24 cells (14), suggesting that this 50-kDa protein is involved in FN-dependent mycobacterial binding to host cells. In this study, we showed that an internal peptide sequence from this 50-kDa protein is homologous to a predicted peptide sequence from an open reading frame (ORF) in an *M. leprae* cosmid and to an *M. leprae* λgt11 expression clone (20). Further, the recombinant protein expressed from the ORF was recognized by a polyclonal antibody against *M. tuberculosis* protein MPT32 and by antibodies present in serum from leprosy patients (25). We expressed this *M. leprae* ORF and showed that a recombinant protein made in *Escherichia coli* binds to FN. We call this FN binding protein the FAP of *M. leprae* (FAP-L). Antibodies to FAP and recombinant FAP-L (rFAP-L) inhibit FN-dependent *M. leprae* uptake by both the T-24 bladder carcinoma cell line and the JS-1 Schwannoma cell line, demonstrating that FAP is necessary for interaction of *M. leprae* with FN and thus for cellular uptake of bacteria. These data identify FAP-L as a necessary component of *M. leprae* binding to FN and suggest that the highly conserved FAP homologies in other mycobacteria have a similar function. Although the means by which *M. leprae* survives within nonmacrophage cell types in vivo are unknown, our data suggest that *M. leprae* interaction with FN is an important step in the attachment and invasion of epithelial and Schwann cells by *M. leprae* and that FAP is an important component of this process.

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## MATERIALS AND METHODS

**Purification of *M. vaccae* FAP.** The 50-kDa FAP of *M. vaccae* was purified from *M. vaccae* culture supernatants as previously described (19), with some modifications to give a higher yield of pure FAP. Briefly, *M. vaccae* supernatant proteins were precipitated with $(NH_4)_2SO_4$ at 50% saturation. The sample was dialyzed against 20 mM Tris-HCl (pH 6.5) and then fractionated on DEAE-Sephacel by using a 0 to 0.4 M linear NaCl gradient. The FAP eluted at 150 mM NaCl, and these FAP-containing fractions were pooled, dialyzed against 20 mM Tris buffer (pH 7.25), and subsequently fractionated on a Mono-Q high-pressure liquid chromatography (HPLC) column by using the same NaCl gradient as described above. FAP eluted from the Mono-Q column at 150 mM NaCl and was dialyzed against 20 mM ammonium acetate ($NH_4OAc$) (pH 6.5). This was followed by separation on a C4 reverse-phase HPLC column using a linear gradient of 20 to 100% acetonitrile–20 mM $NH_4OAc$ (pH 6.5). The FAP eluted at 60% acetonitrile and was dialyzed against 20 mM Tris buffer (pH 7.0). The purified FAP showed a single band at 50 kDa on silver-stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Purified FAP gave no sequence by Edman degradation; therefore, the protein was incubated with cyanogen bromide in 70% formic acid for 24 h and the resultant peptide fragments were separated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane, (Millipore, Bedford, Mass.) and the peptide sequence was deduced from a single band. Rabbit polyclonal antibodies to the FAP of *M. vaccae* were generated by immunizing a rabbit with the purified protein in alum; immunoglobulin G (IgG) was purified from immune serum by protein A-Sepharose chromatography.

**Anti-FAP monoclonal antibodies.** Lou rats (Harlan Sprague Dawley, Inc., Indianapolis, Ind.) were injected in the footpads with 15 μg of purified *M. vaccae* FAP emulsified in Hunter titermax adjuvant (Sigma, St. Louis, Mo.). The rats were boosted with 5 μg of FAP in Hunter titermax adjuvant on day 20. The rats were injected intravenously with 2 μg of FAP in 0.9% sodium chloride 20 days after the second footpad injection and 3 days prior to isolation of the spleen cells and fusion with the murine nonsecreting meloma cell line P3X63Ag8.653. Hybridoma culture supernatants were screened by enzyme-linked immunosorbent assay (ELISA) against the purified *M. vaccae* FAP. Culture supernatants from subclones of the positive hybridomas were again screened by ELISA and by immunoprecipitation of *M. vaccae* FAP and rFAP-L. Three of the six IgG-producing subclones generated produced antibodies which recognized both *M. vaccae* FAP and rFAP-L. Culture supernatant from subclone 2E5 was used in this study.

**Southern blotting.** A 787-bp PCR product of the FAP-L ORF was amplified from the B-38 cosmid clone by using PCR primers 5′-GATAGACTCAGCCA GCTC-3′ (upstream) and 5′-ACTCGACACATCGCAAAG-3′ (downstream). The mycobacterial genomic DNA was isolated (11) and completely digested with *Pst*I, separated on a 1% agarose gel, and transferred to a Nytran nylon membrane (Schleicher & Schuell, Inc., Keene, N.H.). The blot was probed with a $^{32}$P-labeled PCR fragment in accordance with the manufacturer's instructions. The filter was sequentially washed for 30 min each time in 1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 25°C, 1× SSC at 42°C, and 0.1× SSC at 42°C.

**Expression and purification of rFAP-L.** The expression clone for rFAP-L was made as follows. A 1.8-kb *Pst*I-*Kpn*I fragment containing the ORF was subcloned from the 42-kb B-38 cosmid clone into pTrcHisC (Invitrogen, San Diego, Calif.). Low-level expression of FAP-L was obtained in *E. coli* cells transformed with the pTrcHis 1.8-kb *M. leprae* DNA clone after 3 h of incubation with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG; Gibco BRL, Gaithersburg, Md.). To remove the bases 5′ of the ORF and make a fusion protein, the clone was partially digested with *Bgl*I, which cuts 65 bp into the ORF. The correct linearized clone was digested with *Bgl*II (just 5′ of the *Pst*I site within the vector) and blunted with Klenow, and the clone lacking the 187 bp between the *Bgl*II and *Bgl*I sites was religated. *E. coli* XL1-B cells were transformed with the pTrcHisC or the pTrcHis–FAP-L construct. Expression of the FAP-L fusion protein was induced by addition of IPTG to a final concentration of 1 mM, and the protein was purified by $Ni^{2+}$ metal ion affinity chromatography in accordance with the manufacturer's protocol. The bound protein was eluted in 20 mM phosphate buffer (pH 4.0) and immediately neutralized with phosphate buffer. The cell lysates or purified FAP-L were visualized by Coomassie blue-stained SDS-PAGE and Western blotting (immunoblotting). For Western blots, proteins were transferred to Immobilon P (Millipore) and probed with the antibodies indicated in the figure legends. Bound antibodies were ascertained by using protein A-horseradish peroxidase (HRP; Bio-Rad, Richmond, Calif.) for the polyclonal anti-FAP antibody and anti-rat IgG-HRP (ICN Biomedicals, Costa Mesa, Calif.) for the monoclonal anti-FAP antibody. The bound HRP was detected with the ECL detection system (Amersham, Arlington Heights, Ill.).

**FN binding assay.** Ninety-six-well Immulon plates (Dynatech, Chantilly, Va.) were coated overnight at 25°C with 6 μg of a carboxyl-terminal heparin binding chymotryptic fragment of human FN or with bovine serum albumin (BSA). The FAP-L fusion protein was purified as described above and biotinylated with NHS-LC-Biotin (Pierce, Rockford, Ill.). Ten micrograms of affinity-purified anti-FN polyclonal antibody per well (14) was incubated in the FN-coated wells for 1 h, or 20 μg of anti-FAP IgG or control rabbit IgG (Sigma) was incubated with each concentration of the FAP-L fusion protein in phosphate-buffered saline (PBS) for 1 h at 25°C prior to the addition of rFAP-L to coated wells. After 2 h of incubation at 25°C, the wells were washed with PBS–0.1% BSA–0.05% Tween 20 (wash buffer) and the bound biotinylated FAP-L fusion protein was detected with streptavidin-HRP (Research Diagnostics Inc., Flanders, N.J.) and *o*-phenylenediamine dihydrochloride (Sigma) as the substrate for HRP.

The 40- and 50-kDa species of rFAP-L were separated with a Superose 6 gel permeation column (Pharmacia LKB Biotechnology, Uppsala, Sweden). Two hundred micrograms of $Ni^{2+}$ affinity-purified FAP-L in phosphate buffer (pH 7.0) was added to the Superose 6 HPLC column equilibrated in PBS. The 0.5-ml factions collected were analyzed by SDS-PAGE to define the fractions containing FAP-L. Those fractions containing only the 40-kDa FAP-L or only the 50-kDa FAP-L as defined by SDS-PAGE were used in subsequent FN binding experiments. The HPLC-purified rFAP-L proteins were added to wells of a 96-well Immulon plate coated with 6 μg of recombinant FN or with BSA. The FAP proteins were incubated in the FN-coated wells for 2 h at 25°C. The wells were washed with wash buffer, and the bound FAP was detected with a 1/1,000 dilution of anti-FAP polyclonal serum diluted in wash buffer. The bound antibody was detected with protein A-HRP (Sigma) and *o*-phenylenediamine dihydrochloride as the substrate for HRP.

***M. leprae* attachment and ingestion by Schwann and epithelial cells.** T-24 cells ($5 \times 10^4$) in complete medium (IMDM [Gibco BRL] containing 10% fetal calf serum) were plated into each well of a four-chamber Lab-Tek Chamber Slide (Nunc Inc., Naperville, Ill.) precoated with 100 μg of laminin per ml and used approximately 9 h after plating. Irradiated *M. leprae* was isolated as previously described (11) and fluorescein isothiocyanate (FITC) labeled by incubation with 10 mg of FITC (Molecular Probes, Inc., Eugene, Oreg.) in 0.45 M boric acid–0.18 M NaCl–10% dimethyl sulfoxide (pH 9.5) at 25°C for 2 h. The FITC-labeled *M. leprae* was washed and resuspended in Tris buffer (pH 6.0). For FN opsonization, 0.2 ml of 50 mM Tris buffer (pH 6.0) containing $5 \times 10^5$ irradiated *M. leprae* cells was incubated at 37°C for 1 h with or without 400 μg of human FN per ml prior to 1:1 dilution of the *M. leprae* with IMDM (Gibco) and its addition to the T-24 cells. For inhibition studies, *M. leprae* was incubated with 100 μg of anti-FAP or control rabbit IgG per ml for 1 h prior to addition of FN, or FN was incubated with 50 μg of affinity-purified anti-FN antibody per ml, 150 μg of rFAP-L per ml, or an equal volume of a control $Ni^{2+}$ affinity elution (see Fig. 3A, lane 3) for 1 h prior to addition of *M. leprae*. These concentrations of FN and potential inhibitors were determined to be optimal in preliminary experiments.

Subsequent to the above-described incubations, the T-24 cells were washed with PBS and *M. leprae* was added. After 3 h of infection, the T-24 cells were washed with PBS to remove unattached *M. leprae*. T-24 cells with attached and/or phagocytosed *M. leprae* were scored by visual inspection with a Nikon Microphot-FX fluorescence microscope (6). Similarly, JS1 Schwannoma cells at a concentration of $5 \times 10^4$ per well in Dulbecco modified Eagle medium–10% fetal calf serum were plated onto a laminin-coated surface (14), allowed to attach for 4 h, and then incubated with FITC-labeled, irradiated *M. leprae* which had been incubated with FN and/or potential inhibitors of attachment and uptake as described above. The numbers of T-24 and Schwann cells with internalized *M. leprae* were determined with a double-immunofluorescence assay as previously described for T-24 cells and BCG (14). Briefly, the cells were incubated with $5 \times 10^5$ unlabeled *M. leprae* cells for 3 h with or without FN and potential inhibitors. The cells were then washed and incubated with polyclonal rabbit anti-BCG antibody and then with FITC-conjugated goat anti-rabbit IgG to detect extracellular *M. leprae*. Subsequently, intracellular *M. leprae* was detected by permeabilizing the cells with methanol and labeling the mycobacteria with anti-BCG and rhodamine-5-isothiocyanate (RITC)-conjugated anti-rabbit IgG. Only those cells containing *M. leprae* monolabeled with RITC were defined as having internalized mycobacteria. The internalization assay was verified by electron microscopy (14). Where indicated, data are presented as the mean ± the standard error of the mean of three independent determinations. Statistical analysis was performed with the Student paired *t* test.

## RESULTS

**Cloning of FAP-L.** To define the role of FAP in *M. leprae* invasion of Schwann cells and epithelial cells, we cloned the gene that encodes FAP-L. The peptide sequence from a CNBr digestion of purified *M. vaccae* FAP was found to be 73.7% identical to a predicted 19-amino-acid sequence in the translation of an *M. leprae* ORF in cosmid B-38 (GenBank accession no. L01095) (Fig. 1A). The complete ORF containing this peptide sequence encodes a protein with a predicted molecular mass of 29.5 kDa with an amino-terminal stretch of 39 amino acids consistent with a secretory signal (24) (Fig. 1B). The predicted molecular mass of the mature protein is 25.5 kDa. The gene was found to be essentially identical to an *M. leprae* gene originally selected from an *M. leprae* λgt11 expression library by using pooled serum samples from leprosy patients (20). The predicated proteins differ at amino acids 33 and 34

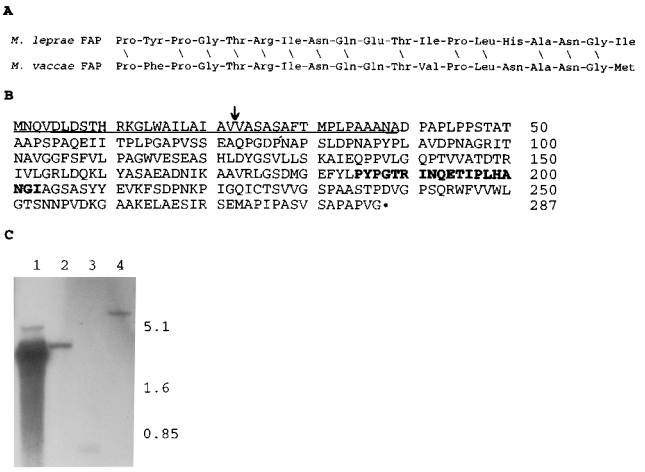


FIG. 1. Protein sequence of FAP-L. (A) The peptide sequence contained within FAP-L aligned with the CNBr peptide fragment from purified *M. vaccae* FAP. (B) Translation of the *M. leprae* ORF which encodes the FAP-L contained within 42-kb *M. leprae* cosmid clone B-38. The ORF predicts a protein of 29.5 kDa, which has a putative signal sequence (underlined). The size of the predicted mature protein is 25.5 kDa. The peptide sequence from panel A is shown in bold type. The arrow indicates the 22 amino acids removed in the expression of the rFAP-L fusion protein. (C) Southern blot of mycobacterial DNA probed with a PCR-synthesized 787-bp fragment from the FAP-L ORF. Lanes 1 to 4 were loaded with 3 μg of *Pst*I-digested *M. leprae*, *M. avium*, *M. tuberculosis*, and *M. vaccae* genomic DNAs, respectively. Sizes are indicated in kilobases.

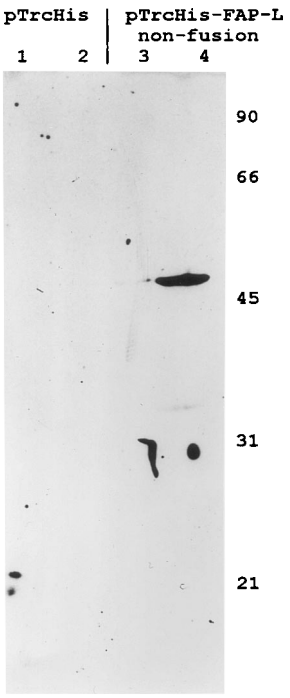


FIG. 2. *E. coli*-expressed FAP-L is recognized by anti-FAP antibody. pTrcHis with the cloned 1.8-kb *M. leprae Pst*I-*Kpn*I DNA fragment containing the FAP-L ORF was used in expression studies as described in Materials and Methods. Lanes 1 and 2, contained protein lysates from *E. coli* transformed with pTrcHisC. Lanes 3 and 4 contained protein lysates from *E. coli* transformed with the pTrcHis–1.8-kb *M. leprae* DNA clone. Lanes: 1 and 3, total cell lysate of transformed *E. coli* without IPTG induction; 2 and 4, cell lysates from transformants treated with 1 mM IPTG for 3 h. The proteins were run on SDS–10% PAGE under reducing conditions, transferred onto Immobilon P membranes, and probed with anti-FAP rabbit serum. The relative molecular masses of protein standards are indicated in kilodaltons.

with a Leu-Pro in the translated sequence from the cosmid clone and a Phe-Arg in the 43L protein (25). These differences may represent polymorphism in the *M. leprae* gene. A 787-bp PCR fragment covering a portion of this ORF when used to probe a Southern blot containing *Pst*I-digested *M. avium*, *M. vaccae*, *M. tuberculosis*, and *M. leprae* DNAs showed a single cross-hybridizing band (Fig. 1C), demonstrating conservation of this gene throughout the genus *Mycobacterium*. To determine whether this protein is the *M. leprae* homolog of the *M. vaccae* FN binding protein (19), the full-length FAP-L clone was expressed in *E. coli* (Fig. 2). Expression of a 50-kDa protein which reacted with a polyclonal antibody to *M. vaccae* FAP was induced in *E. coli* which had been transformed with the FAP-L ORF. Thus, the cloned ORF encodes a protein with antigenic and sequence similarity to *M. vaccae* FAP. We call this *M. leprae* protein FAP-L because it is the *M. leprae* homolog of the uncloned *M. vaccae* protein. The migration of FAP-L at 50 kDa on SDS-PAGE was unexpected since the predicted mass of the protein was 29.5 kDa. We resequenced the clone and confirmed the stop codon found in the original sequencing of the B-38 cosmid clone. This suggests that FAP has an unusual migration on SDS-PAGE, perhaps because of its high proline content. Indeed, analysis of individual FAP domains expressed in *E. coli* showed that these also migrated anomalously slowly on SDS-PAGE (data not shown). Interestingly, FN binding protein Sfb from *Streptococcus pyogenes*, which also contains a large number of proline residues, migrates on SDS-PAGE at approximately twice its predicted molecular mass of 71 kDa, similar to what was seen with FAP-L (23).

**Binding of rFAP-L to FN.** To prove that the protein encoded by the *M. leprae* ORF binds FN, we attempted to purify rFAP-L from *E. coli*. However, expression of this protein was too inefficient for purification of the quantities needed for functional studies. Therefore, we synthesized a fusion protein consisting of amino acids 23 to 288 of FAP-L with a His-rich amino-terminal peptide to aid protein purification by subcloning a *Bgl*I-*Kpn*I fragment of the FAP-L gene into pTrcHisC (Fig. 3). When the fusion protein was expressed and purified, there were two predominant bands on SDS-PAGE (Fig. 3A), at 40 and 50 kDa, the larger of which comigrated with purified native *M. vaccae* FAP. Both bands reacted with polyclonal and a monoclonal antibodies to *M. vaccae* FAP on a Western blot (Fig. 3B and C, respectively). Since the lower-$M_r$ band increased with time prior to purification and because expression of full-length FAP-L with its own leader sequence and without the fusion peptide from the pTrcHis vector led to expression of only the 50-kDa band (Fig. 2), we suspect that the 40-kDa band represents a proteolytic fragment of the 50-kDa FAP-L fusion protein. Further, this is in agreement with the expression of a β-galactosidase fusion protein containing the FAP-L ORF which produced a doublet of 158 and 153 kDa in *E. coli* (48 and 43 kDa minus β-galactosidase) (20). However, this is not in agreement with a pGEX fusion protein which produced a doublet of 31 and 27 kDa after cleavage of the fusion partner (25). The reason for this discrepancy in the $M_r$ of FAP-L on SDS-PAGE is unclear, but it may represent differences in susceptibility of the fusion protein to degradation in different *E. coli* strains. Purified rFAP-L fusion protein bound plasma FN (data not shown) and the carboxy-terminal heparin binding chymotryptic fragment of FN (Fig. 4A). We and others have found that the carboxy-terminal heparin binding domain of FN is the site to which mycobacteria adhere (8; unpublished data). The binding of the fusion protein to FN was blocked with antibodies to both FN and *M. vaccae* FAP (Fig. 4A). The

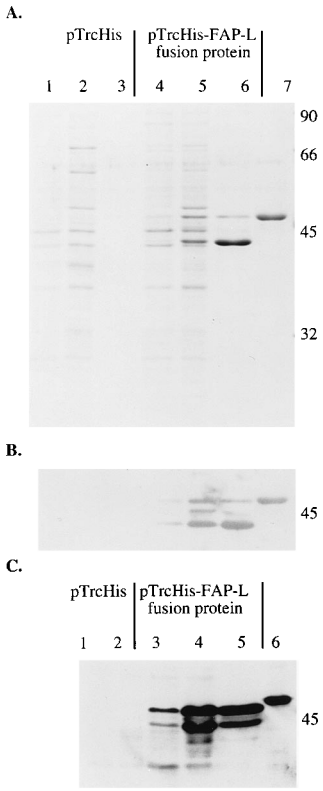


FIG. 3. Expression and purification of a FAP-L fusion protein. (A) Expression and purification of rFAP-L. The fusion protein was made as described in Materials and Methods and lacks the N-terminal 22 amino acids of the complete FAP-L sequence (arrow in Fig. 1B) but has an additional 38 N-terminal amino acids contributed by the vector. Shown are lysates and proteins eluted from the $Ni^{2+}$ affinity column run under reducing conditions on SDS–10% PAGE and stained with Coomassie blue. Lanes: 1 to 3, proteins from pTrcHisC-transformed *E. coli*; 4 to 6, proteins from pTrcHis–FAP-L-transformed *E. coli*; 1 and 4, total lysates from transformed *E. coli* without IPTG induction; 2 and 5, lysates from transformants treated with 1 mM IPTG for 3 h; 3 and 6, proteins eluted from an $Ni^{2+}$ affinity column (equal volumes of elution pools were loaded); 7, 2 μg of purified *M. vaccae* FAP. The relative molecular masses of protein standards are indicated in kilodaltons. (B) Recognition of rFAP-L by a polyclonal antibody made against *M. vaccae* FAP. A gel identical to that in panel A was transferred onto an Immobilon P membrane and probed with anti-FAP rabbit serum. (C) Recognition of rFAP-L by a monoclonal antibody made against *M. vaccae* FAP. *E. coli* lysate and purified rFAP-L were resolved by SDS-PAGE, transferred onto Immobilon P, and probed with a 1/50 dilution of anti-FAP monoclonal antibody 2E5 culture supernatant. Lanes: 1 and 2, proteins from *E. coli* cells transformed with pTrcHis; 3 and 4, lysates from pTrcHis–FAP-L-transformed *E. coli*; 1 and 3, total lysates before IPTG induction; 2 and 4, total lysates after IPTG induction; 5, 1 μg of rFAP-L eluted from an $Ni^{2+}$ affinity column; 6, 1 μg of purified *M. vaccae* FAP.

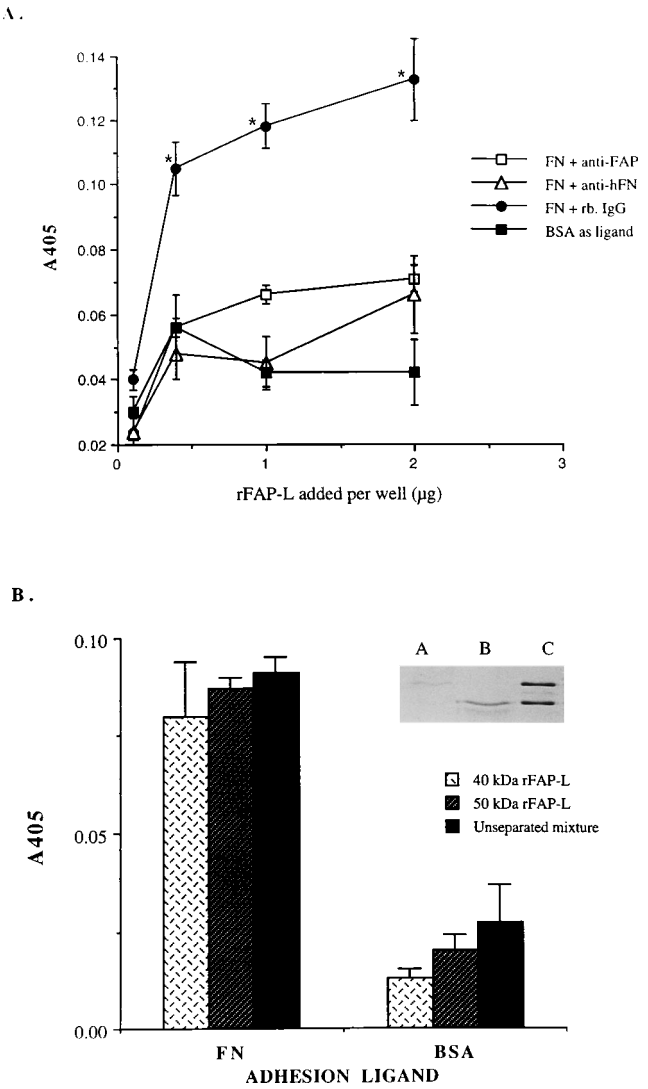


FIG. 4. Binding of purified rFAP-L to FN. (A) Inhibition of FAP-L binding to FN by anti-FN and anti-FAP antibodies. Biotinylated rFAP-L was incubated in FN- or BSA-coated wells with and without antibodies. Each point is the mean ± the standard error of the mean of triplicate wells. The asterisks show values significantly different from those of BSA-coated wells at $P < 0.05$ (Student paired *t* test). (B) Binding of human FN by both the 50- and 40-kDa FAP-L forms. Size-separated 50- and 40-kDa FAP-L fusion proteins and unseparated FAP-L were incubated in FN- or BSA-coated wells (0.2 μg per well) for 2 hours at 25°C. Bound FAP-L was detected by ELISA with the anti-FAP polyclonal antibody. Each point is the mean ± the standard error of the mean of triplicate wells. Inset: Coomassie blue staining of separated 40- and 50-kDa fusion proteins. Lanes: A, 50-kDa pool; B, 40-kDa pool; C, recombinant protein pool from an $Ni^{2+}$ affinity column, which was the starting material for the separation.

isolated 40- and 50-kDa fusion proteins each bound FN (Fig. 4B). The ability of rFAP-L to bind FN further supports the similarity between the cloned *M. leprae* ORF and the *M. vaccae* FN binding protein.

**FAP-mediated *M. leprae* attachment and invasion of Schwann and epithelial cells.** To determine the role of FAP-L in *M. leprae* binding and invasion of cells, we examined the attachment and uptake of *M. leprae* by T-24 transitional carcinoma cells and by the JS1 Schwannoma cell line. Addition of FN significantly increased *M. leprae* binding to and invasion of both T-24 and JS1 cells (Fig. 5 and 6, respectively). Incubation of *M. leprae* with the polyclonal antibody to *M. vaccae* FAP inhibited the FN-mediated binding to T-24 (Fig. 5A) and JS1 (Fig. 6A) cells. Purified rFAP-L also significantly inhibited *M. leprae* attachment. Anti-FAP and rFAP-L also inhibited *M. leprae* invasion of T-24 and JS-1 cells (Fig. 5B and 6B, respectively). These data show that FAP-L is an FN binding protein which is required for efficient recognition and uptake of *M. leprae* by both epithelial and Schwann cells.

## DISCUSSION

We report here the expression of an *M. leprae* gene which, by sequence homology and antigenic cross-reactivity, is the *M. leprae* homolog of the purified *M. vaccae* FN binding protein which we have previously characterized immunologically (14).

The data support the hypothesis that the interaction of

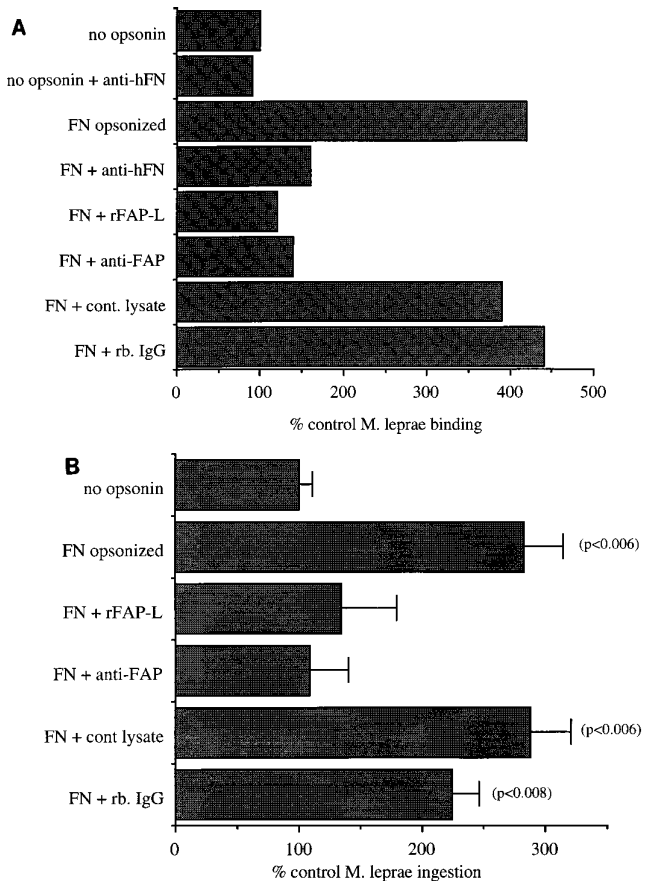


FIG. 5. Inhibition of *M. leprae* attachment and ingestion by T-24 cells with anti-FAP antibody and rFAP-L. (A) FAP-L mediated binding of *M. leprae* to T-24 cells. Incubation of *M. leprae* with T-24 cells with and without FN and potential inhibitors was performed as described in Materials and Methods. The number of T-24 cells with associated *M. leprae* in the absence of FN and inhibitors was recorded as 100% binding and averaged 10 mycobacteria per 100 cells. Data are from a single experiment representative of three. (B) FAP-L mediated ingestion of *M. leprae* by T-24 cells. Detection of T-24 cells with internalized *M. leprae* was determined with a double-immunofluorescence assay. The data from three separate experiments were combined by comparing *M. leprae* ingestion in each experimental condition with *M. leprae* ingestion by T-24 cells in the absence of FN and inhibitors, which was labeled 100% and averaged 6.7 ± 0.7 mycobacteria per 100 cells. *P* values of $<0.05$ compared with the no-opsonin control are shown. cont., control; rb., rabbit.

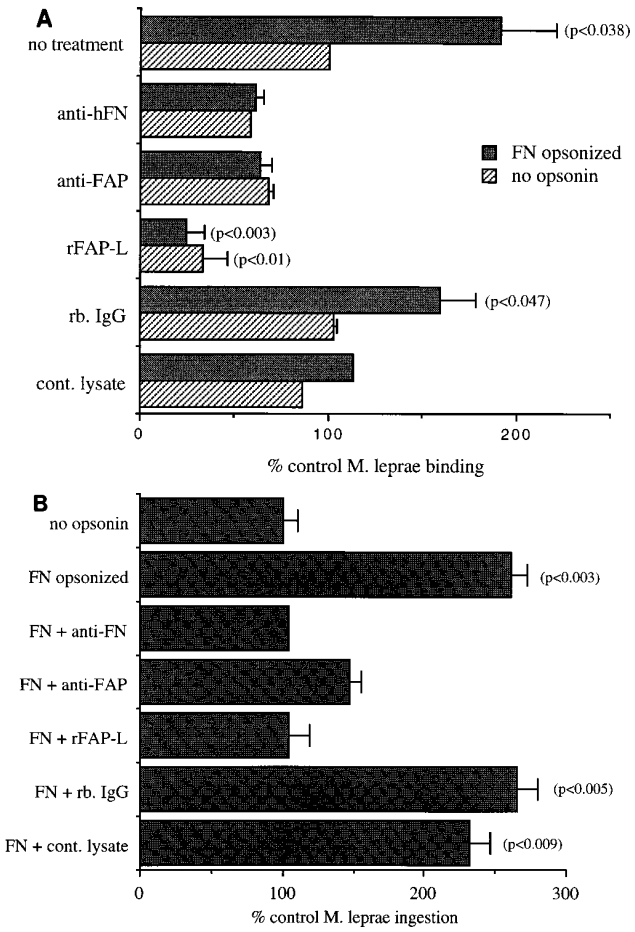


FIG. 6. Inhibition of *M. leprae* attachment and ingestion by JS1 cells with anti-FAP antibody and rFAP. (A) FAP-L-mediated binding of *M. leprae* to JS1 Schwannoma cells. The number of JS1 cells with attached and/or phagocytosed *M. leprae* cells was determined as described in Materials and Methods. The data from three separate experiments were combined by comparing *M. leprae* attachment in each experiment with *M. leprae* attachment to JS1 cells in the absence of FN and inhibitors, which was called 100%. Data are plotted as the mean ± the standard error of the mean. Control binding averaged 25.3 ± 6.6 mycobacteria per 100 cells. The data on JS1 cells treated with the control *E. coli* lysate proteins eluted from an $Ni^{2+}$ affinity column are averages of two experiments. (B) FAP-L-mediated ingestion of *M. leprae* by JS1 Schwannoma cells. The data from three separate experiments were combined as described above for *M. leprae* attachment to JS1 cells. Anti-FN treatment data are averages of two experiments. Control ingestion averaged 3.2 ± 0.33 *M. leprae* organisms per 100 cells. A total of 300 cells were counted per condition for each experiment. Internalized *M. leprae* was defined as mycobacteria available to antimycobacterial antibodies only after permeabilization of the Schwann cells. Incubation of *M. leprae* with FN and potential inhibitors was done as described in Materials and Methods. *P* values of $<0.05$ compared with the no-opsonin control are shown. rb., rabbit; cont., control.

FAP-L with FN is an important step in the pathogenesis of leprosy, since invasion of Schwann cells by *M. leprae* is responsible for peripheral nerve damage, a major manifestation of the disease (3). *M. leprae* appears to use FN as a conventional opsonin. The bacteria bind FN via FAP-L and use eukaryotic cell receptors for FN to bind and enter host cells. This is analogous to the role of complement in the uptake of mycobacteria, including *M. leprae*, by macrophages (10, 15, 22). Macrophages also have FN receptors and may be able to use FN, as well as complement, to opsonize mycobacteria for phagocytosis (5). Since the FAP family is highly conserved among mycobacteria, this cannot be the gene product which makes *M. leprae* unique in its ability to survive within epithelial or Schwann cells. Rather, FAP interaction with FN is an important determinant of mycobacterial entry into cells. Indeed, mycobacterial invasion of Schwann cells is not specific for *M. leprae* (4), suggesting that the invasion mechanism, like FAP, is present in the whole genus.

Several different mycobacterial FN binding proteins have been described. These include the 27- to 32-kDa proteins which are part of the 85ABC complex (26), as well as a 55-kDa *M. tuberculosis* protein (1). The 85ABC complex is not recognized by antibodies to FAP and vice versa (data not shown). The *M. tuberculosis* 55-kDa gene did not hybridize with the FAP-L gene (data not shown), so these also represent independent binding activities. Rao et al. have suggested the presence of a $\beta_1$ integrin on the *M. avium* cell surface which mediates binding to FN (18). Their conclusions were based on immunoblots and the ability of polyclonal anti-human $\beta_1$ to block *M. avium* binding to FN-coated wells. Whether or not this cross-reactive protein is expressed by other mycobacteria is

not known. It is not clear whether there is any role for these FN binding proteins in the recognition and invasion of host cells by mycobacteria. In contrast, anti-FAP blocks the uptake of both BCG and *M. leprae* by host cells. Conservation of the FAP family and of FN binding among mycobacteria suggests that this interaction may be an important step in host cell infection by this entire genus of intracellular pathogens.

It is interesting that FAP-L, also known as L14 (20) and 43L (25), has been shown to elicit a T- and B-cell response in a number of lepromatous and borderline lepromatous leprosy patients (25), suggesting that FAP-L has an immunological, as well as a functional, role in *M. leprae* infection. Further, one could speculate that the ability of FAP-L to bind FN may enhance its attachment and internalization by antigen-presenting cells for subsequent presentation to T cells.

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