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Identification and Analysis of *fipA*, a *Fusobacterium nucleatum* Immunosuppressive Factor Gene

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We have previously demonstrated that sonic extracts of *Fusobacterium nucleatum* FDC 364 were capable of inhibiting human T-cell responses to mitogens and antigens. The purified *F. nucleatum* immunosuppressive protein (FIP) is composed of two subunits of 44 and 48 kDa. Furthermore, FIP inhibits T-cell activation by arresting cells in the middle of the G_1 phase of the cell cycle; the data available to date suggest that FIP impairs the expression of the proliferating-cell nuclear antigen. To initiate delineation of FIP structure-function relationships, molecular cloning of the FIP gene was carried out. A DNA library of *F. nucleatum* FDC 364 was constructed by partial digestion of genomic DNA with *Sau*3A and screened for the production of FIP with polyclonal antibody. Twelve immunoreactive clones were identified. One of these clones contained a 3.1-kbp insert and was chosen for further study. Cell lysates were found to contain an immunoreactive band that comigrated with the 44-kDa subcomponent of the native FIP. Sequencing of the 3.1-kbp insert revealed the presence of three open reading frames (ORFs). One ORF extends from nucleotides 415 to 1620, encodes 402 amino acids, and is preceded by a ribosome-binding site. Deletion analysis and antibody elution analysis showed that this ORF encodes the 44-kDa subunit (FipA) of native FIP. A second ORF is situated upstream of *fipA*. However, Northern (RNA) analysis suggested that *fipA* is not transcribed as part of an operon but is transcribed from its own promotor. Finally, the partially purified recombinant FipA protein was capable of impairing T-cell activation in a manner consistent with the native protein. These results indicate that the two components that form the native protein are most probably distinct gene products and suggest that the 44-kDa FipA polypeptide is sufficient to mediate the immunosuppressive activities of the native protein complex.

Fusobacterium nucleatum is a gram-negative, anaerobic organism that is implicated as an opportunistic pathogen in several disease entities. This species has been observed as the sole infecting organism in several cases of bacteremia (5), urinary tract infection (15), and pericarditis (26). In addition, *F. nucleatum* has been implicated in several pediatric diseases including upper respiratory tract infections and otitis media (2). Furthermore, organisms such as *F. nucleatum* have been reported to act as cofactors for diseases such as Burkitt's lymphoma and nasopharyngeal sarcoma because of their ability to produce metabolic products that may act as inducers of latent viruses such as Epstein-Barr virus (8). Finally, *F. nucleatum* is a suspected pathogen in several oral diseases such as pulp infections, alveolar abscesses, and periodontal disease (17, 24, 28, 29). The importance of *F. nucleatum* in oral disease is of particular significance, because the oral route is the suspected portal of entry of this organism in other diseases. In spite of its implication in numerous disease states, there is little information about how *F. nucleatum* might act to cause disease. In this regard, we have previously demonstrated that soluble sonic extracts of *F. nucleatum* FDC 364 can suppress human lymphocyte proliferative responses to both mitogens and antigens in vitro (22). Suppression was observed to be dose dependent, occurred by noncytotoxic mechanisms, and involved alterations in DNA, RNA, and protein synthesis. We have purified the *F. nucleatum* immunosuppressive protein (FIP); it has an apparent molecular mass of 90 to 100 kDa. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) indicates that FIP is actually composed of two subunits with molecular masses of 48 and 44 kDa; however, it is unclear if these subunits are encoded by separate genes or are the result of protein processing of a single gene product. Studies on the mode of action of FIP suggest that it prevents cells from exiting the G_0/G_1 phase of the cell cycle (21). However, FIP-treated cells were capable of expressing activation markers (CD69, CD25, and CD71) following mitogen activation. Analysis of the expression of cyclins indicates that the phase of the cell cycle that is FIP sensitive resides somewhere beyond the restriction point of cyclin D_2 but prior to that of cyclins D_3 and E. Finally, inhibition of the expression of the proliferating-cell nuclear antigen was found to be the earliest detectable lesion in FIP-treated cells. Collectively, these results indicate that the mechanism by which FIP inhibits lymphocyte activation involves a mid-G_1 block of the cell cycle.

An understanding of how FIP associates with T cells may provide insights into the general biology of lymphocyte activation and, in addition, may further delineate the mode of action of this immunomodulatory protein in cell cycle arrest of human lymphocytes. To address these issues, it will be important to define the molecular interrelationships between the structure of FIP and its biological functions. Cloning of the FIP gene and the use of high-level expression vectors provide an approach for delineating the structure of the FIP as well as providing relatively large amounts of material for the study of its mechanism of action. In the present study, we report the molecular cloning, expression, and sequencing in *Escherichia coli* of the gene encoding the *F. nucleatum* inhibitory protein.

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MATERIALS AND METHODS

Culture conditions. *F. nucleatum* FDC 364 was grown as previously described (22). Briefly, the bacteria were grown for 3 to 4 days at 37°C in brain heart infusion broth (Difco Laboratories, Detroit, Mich.) supplemented with 0.2% yeast extract (Difco), 0.5 mg of L-cysteine hydrochloride, and freshly prepared 0.5% sodium bicarbonate in a hydrogen-carbon dioxide atmosphere. A plasmid library of *F. nucleatum* was constructed with *E. coli* DH5α as the host; these cells were grown at 37°C in SOB medium (20 g of Bacto Tryptone per liter, 5 g of Bacto Yeast Extract per liter, and 0.5 g of NaCl per liter, containing 25 mM KCl and 20 mM $MgCl_2$).

Preparation of polyclonal antiserum to native FIP. New Zealand White male rabbits were immunized with 50 μg of purified native FIP prepared as previously described (21). The protein preparation was emulsified in complete Freund adjuvant. The rabbits were boosted on days 10, 20, and 40 with protein in incomplete Freund adjuvant and bled on day 45. Serum was tested for the presence of anti-FIP antibodies by Western blot (immunoblot).

Construction of *F. nucleatum* genomic library and immunological screening. Genomic DNA was isolated from cultures of *F. nucleatum* FDC 364. Briefly, the bacterial cells were washed and resuspended in 25 ml of 50 mM Tris (pH 8) containing 0.1 M EDTA, 0.15 NaCl, and 0.5 mg of lysozyme per ml. Following incubation for 30 min at 37°C, 2 ml of 25% SDS was added and the cells were incubated at 55°C for 10 min. Genomic DNA was then extracted with phenol-chloroform-iodoacetic acid and precipitated in ethanol. The DNA was then resuspended in 10 mM Tris (pH 8) containing 1 mM EDTA, 50 μg of RNase per ml, and 50 μg of proteinase K per ml. Following additional extraction with phenol-chloroform, the DNA was partially digested with *Sau*3A. Fragments were size fractionated on a 10 to 40% sucrose gradient by centrifugation for 16 h at 150,000 × *g* and analyzed by agarose gel electrophoresis. Fractions containing DNA fragments of 2 to 6 kbp were pooled and recovered by ethanol precipitation. The fragments were then ligated into pUC19 that had been cleaved with *Bam*HI and transformed into *E. coli* DH5α. Recombinant colonies were transferred to nitrocellulose and lysed by exposure to chloroform vapor for 15 min followed by treatment (2 h) with lysis buffer (phosphate-buffered saline [PBS] containing 50 mM $MgCl_2$, 10 μg of DNase per ml, and 0.4 mg of lysozyme per ml). The filters were washed several times, blocked in PBS containing 0.2% Tween 20, and then exposed to rabbit polyclonal anti-FIP serum overnight. After being washed, the filters were treated for 4 h with anti-rabbit immunoglobulin conjugated to horseradish peroxidase and then developed with 4-chloro-1-naphthol in Tris (pH 7.5) containing 0.03% H_2O_2. Positive clones were picked and purified to homogeneity by several cycles of plating and assay.

Characterization, restriction enzyme analysis, and DNA sequencing of recombinant clones. Cell lysates were prepared from colonies which were immunoreactive with the anti-FIP serum. Briefly, 4-ml cultures were grown in SOB medium containing 20 mM $MgSO_4$ and 100 μg of ampicillin per ml with or without 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Cells were then disrupted by sonication on ice. The sonicated cell suspensions were analyzed by SDS-PAGE (12% polyacrylamide gels) and Western immunoblotting onto nitrocellulose (21).

Restriction digests were performed as specified by the manufacturer, with double digests carried out sequentially. The restricted DNA was electrophoresed in 0.8% low-melting-point agarose, and when necessary, regions containing the desired fragments were excised and gel slices were melted in 0.3 M sodium acetate and phenol extracted. The DNA was recovered by ethanol precipitation. After cloning of the DNA into M13mp18 and M13mp19 that had been cleaved with the appropriate restriction endonucleases, nucleotide sequencing was performed by using Sequenase version 2.0 (United States Biochemical) as specified by the manufacturer. Some sequence was carried out with synthetic primers derived from the *fipA*-specific sequences already determined. Both strands of *fipA* were sequenced.

Construction of probes and Northern blot analysis. Probes for Northern (RNA) blot analysis were prepared by PCR amplification of regions within the *fipA* gene as well as from the upstream open reading frame (ORF). To generate the *fipA*-specific probe, we used oligonucleotide primers a and b (5′-GGTAAT GCTTCTGGACT-3′ and 5′-CAGAAGCTCCAACTGGATGTC-3′, respectively). These primers anneal to residues 1157 to 1174 and 1479 to 1501 of the *fipA* sequence, respectively, and amplify a 344-bp product. The probe for the upstream ORF was generated with primers c and d (5′-ATGTCTAAAALTTG GGC-3′ and 5′-TGAAGACTCATCALTTGC-3′, respectively); these primers anneal to nucleotides 114 to 130 and 300 to 317, respectively, in the sequence shown in Fig. 3 and amplify a 203-bp product. Amplification was carried out on a Perkin-Elmer thermocycler with *Taq*I polymerase by following the protocol supplied by the manufacturer. The probes were isolated from agarose gels and end labeled with [^{32}P]ATP by using polynucleotide kinase.

Total RNA was isolated from 250 ml of log-phase *F. nucleatum* FDC 364 cultures by the method of Reddy et al. (14). Cultures were initially put on ice, and 25 ml of 200 mM Tris (pH 8.0) containing 20 mM EDTA and 20 mM sodium azide was added. The cultures were centrifuged, and the cell pellet was suspended in 3 ml of 10 mM Tris containing 400 μg of lysozyme per ml. The cultures were then incubated for 30 min, and 2% SDS was added; this was followed by incubation for 15 min at 60°C. Cell suspensions were extracted with equal volumes of phenol-chloroform, and nucleic acids were precipitated with ethanol. The nucleic acid pellet was suspended in 3 ml of distilled water containing 10 mM vanadyl-ribonucleoside complex and extracted and precipitated as above. The resulting pellet was suspended in 6 ml of H_2O containing 4.6 g of CsCl, layered onto a 3-ml cushion of 5.7 M CsCl, and centrifuged at 100,000 × *g* for 18 h. The RNA pellets were then suspended in 1 ml of distilled water and precipitated several times with ethanol.

For Northern analysis, 20-μg samples of RNA were electrophoresed by established procedures in 1.2% agarose gels containing 3% formaldehyde and transferred to nitrocellulose (18). Hybridizations were carried out with 50% formamide–6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–5× Denhardt's solution (1× Denhardt's solution is 0.1% Ficoll, 0.1% polyvinylpyrrolidone, plus 0.1% bovine serum albumin [BSA])–100 μg of salmon sperm DNA per ml–0.1% SDS at 42°C. Blots were exposed to one of the two probes prepared above.

Affinity purification of anti-recombinant FIP antibody with nitrocellulose blots. Monospecific anti-recombinant FIP was prepared by using preparative immunoblots (12). Immunoblots were prepared as described above. After incubation with primary antibody, the leftmost and rightmost lanes were excised and probed with indicator antibody to determine the locations of the recombinant peptide band. The two pieces of nitrocellulose were then used to approximate the location of the peptide band in the remaining part of the blot. A 3-mm nitrocellulose strip containing the immobilized peptide was excised, and elution of the antibody to the recombinant peptide was carried out for 5 min in 1.5 ml of 3 M KSCN. Control elutions were carried out on regions of the blot which contained unrelated *E. coli* proteins. The supernatants were decanted and dialyzed against PBS. All procedures were carried out at 0 to 4°C. The eluted antibodies were then used to probe immunoblots of native *F. nucleatum* FIP.

Analysis of recombinant FIP for biological activity. Recombinant FIP was prepared from 1-liter cultures of clone 13A′. Briefly, the bacteria were grown overnight at 37°C in SOB containing 20 mM $MgSO_4$. The soluble protein fraction was isolated from bacterial cells that were harvested, washed in PBS, and disrupted by sonication at 4°C. Cellular debris was removed by centrifugation at 12,000 × *g* for 20 min, and the membrane fraction was sedimented by centrifugation at 85,000 × *g* for 60 min. The protein that remained in suspension after the high-speed centrifugation was designated the cytoplasmic fraction and contained both cytoplasmic and periplasmic proteins. The cytoplasmic fraction was first fractionated by ammonium sulfate precipitation, and fractions were monitored by immunoreactivity as described above; all activity precipitated between 60 and 80% $(NH_4)_2SO_4$. Following dialysis against 10 mM Tris buffer (pH 7.0) containing 10 mM NaCl and 1 mM EDTA, the sample was applied to an ion-exchange column (Mono Q10; Pharmacia, Uppsala, Sweden) preequilibrated in this buffer. The column was then extensively washed and eluted with a linear NaCl gradient (10 to 600 mM). Fractions were collected and monitored for A_{280}, for FIP immunoreactivity by Western blot analysis, and for biological activity (see below). Active fractions were pooled and concentrated for further fractionation by gel filtration chromatography on a Superose 12 column (Pharmacia). All fractions were monitored for A_{280}, FIP immunoreactivity, and biological activity. It should be noted that in all instances, the elution profile of biological activity (suppression of lymphocyte proliferation) and immunoreactivity to the anti-FIP sera were identical. Preparations were assessed for purity by SDS-PAGE, and the protein concentration was determined (bicinchoninic acid protein assay; Pierce, Rockford, Ill.) with BSA as a standard.

Human peripheral blood mononuclear cells (HPBMC) were prepared as described previously (22). HPBMC suspension (0.1 ml) containing 2×10^5 cells was placed into each well of flat-bottom microculture plates. Each culture received 0.1 ml of medium or 0.1 ml of different concentrations of recombinant FIP diluted in medium. The cells were then incubated for 30 min at 37°C, at which time the cell cultures received an optimal mitogenic dose of phytohemagglutinin (PHA; 1 μg/ml; Murex Diagnostics, Atlanta, Ga.). The cells were incubated for 96 h, and DNA synthesis was assessed by the incorporation of [^{3}H]thymidine as previously described (22).

Fluorescence-activated cell sorter analysis of activation markers. HPBMC (5×10^6/ml) were incubated for 24 h with PHA in the presence or absence of recombinant FIP. The cells were washed three times with PBS containing 2% fetal bovine serum and stained with anti-CD25 or anti-CD69 monoclonal antibodies conjugated to fluorescein isothiocyanate (FITC; Becton Dickinson Immunocytometry Systems, San Jose, Calif.). The cells were fixed in 1% paraformaldehyde and analyzed on a Becton Dickinson FACStar^{Plus} flow cytometer (23). Appropriate isotype-conjugated control antibodies were used (Becton Dickinson Immunocytometry Systems). Cells which gave fluorescence signals brighter than that observed for 98% of appropriate control cells were considered to be positive; 10,000 cells were analyzed per sample.

Cell cycle analysis. Cell cycle analysis was performed on HPBMC as previously described (21). Briefly, 1-ml cultures (2×10^6 cells) were incubated for 72 h in the presence of medium (control), PHA, or PHA plus recombinant 44-kDa protein (rFipA). Cells were harvested at 24-h intervals, washed (PBS containing 0.1% sodium azide), and then stained with anti-CD3 (or control antibody) conjugated to FITC. The cells were washed and fixed for 60 min with cold 0.25% paraformaldehyde. After being washed, the cells were permeabilized with 0.2% Tween 20 in PBS for 15 min at 37°C. DNA was then stained by incubating the cells with 7-amino-actinomycin (7-AAD; 25 μg/ml) for 30 min. Samples were analyzed on a Becton-Dickinson FACStar^{Plus} flow cytometer equipped with

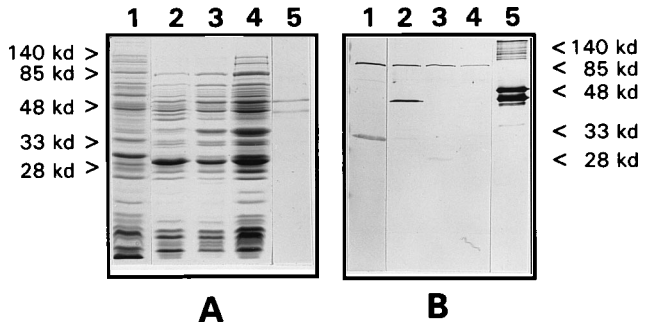

FIG. 1. Immunoblot analysis of protein produced by immunoreactive clones. Bacterial cultures were grown to log phase, and lysates prepared. The lysates were subjected to SDS-PAGE followed by blotting onto nitrocellulose and reaction with anti-FIP serum. (A) SDS-PAGE followed by staining with Coomassie brilliant blue; (B) Western immunoblot. Lanes: 1, clone 14; 2, clone 13A′; 3, clone 13A′-1 kbp (1-kbp fragment generated from digestion of clone 13A′ insert with *Puv*II and *Hin*dIII); 4, pUC19; 5, purified native FIP.

pulse-processing capability for doublet discrimination. FITC and 7-AAD fluorescences were excited by an argon laser operating at 488 nm, and fluorescence was measured with a 530/30-nm bandpass filter and a 650-nm long-pass filter, respectively. A minimum of 50,000 events were collected on each sample. Immunofluorescence was detected by using log amplification, and 7-AAD emissions were collected by using linear amplification; data were analyzed with appropriate software (Modfit; Verity Software House, Topsham, Maine).

Nucleotide sequence accession number. The *fipA* sequence has been submitted to the GenBank database; the accession number is U37723.

RESULTS

Characterization of the *fipA* gene. Polyclonal antiserum to FIP was prepared in rabbits; the antiserum recognizes the two peptides (48 and 44 kDa) that constitute the native FIP (Fig. 1, lanes 5) (21). This antiserum was used to screen expression libraries contained in pUC19. Initial screening of approximately 10,000 colonies yielded 15 clones, of which 12 remained positive after a secondary screen. Restriction analysis suggested that two classes of clones had been identified (results not shown). One group of clones (class I) contained common *Eco*RI-*Hin*dIII fragments of 1.5, 0.8, 0.7, and 0.5 kbp. The second group of clones (class II) contained no *Eco*RI or *Hin*dIII sites. Furthermore, analysis of cell protein derived from lysates of the two classes of recombinant clones showed that class I clones (represented by clone 14) contained an immunoreactive band with a molecular mass of 30 kDa while the class II clones (represented by clone 13A′) expressed an immunoreactive band of approximately 44 kDa that comigrated with the lower band of the native FIP (Fig. 1). A second, 68-kDa species was also weakly recognized by the antiserum; however, this immunoreactive peptide was found in both class I and II clones and in control lysates prepared from pUC19. Clone 13A′ was chosen for further study because it not only expressed a 44-kDa peptide but also reacted most strongly with anti-FIP antibodies and exhibited biological activity similar to the native FIP (see below).

As shown in Fig. 2, the plasmid contained by clone 13A′ (pFIPA) possessed a 3.1-kbp insert and expressed a 44-kDa peptide in the absence of IPTG induction of the vector-derived *lac* promoter. This suggests that clone 13A′ may possess the endogenous *F. nucleatum* promoter. To localize the FIP gene within the insert, restriction fragments generated from digestions of pFIPA with *Hin*dIII and *Nde*I or with *Puv*II and *Hin*dIII were subcloned into pUC19 and analyzed for the production of immunoreactive protein. As shown in Fig. 2, neither the 2.1- nor the 0.9-kbp *Hin*dIII-*Nde*I fragments encoded peptides that were immunoreactive with anti-FIP antibodies. However, subclones containing the 1-kbp *Puv*II-*Hin*dIII fragment (13A′-1 kbp) expressed an immunoreactive peptide of approximately 25 kDa, suggesting that the FIP gene is oriented and situated as shown in Fig. 2.

The sequence of the insert contained in clone 13A′ is presented in Fig. 3; three ORFs were identified in the insert. One ORF extends from residues 415 to 1620, encodes 402 amino acids, and is preceded by a ribosome-binding site. Deletion analysis (see above) and antibody elution analysis showed that this ORF encoded the 44-kDa subunit (FipA) of native FIP. A second ORF (ORF1) is situated upstream of *fipA* and terminates at nucleotide 362. A short intergenic region of 52 nucleotides lies between this ORF and *fipA*. Partial sequence analysis downstream of *fipA* suggests that a third ORF, which is transcribed from the opposite strand, is possible.

The relatively short intergenic domain between ORF1 and *fipA*, as well as the lack of a rho-independent transcriptional termination sequence, suggested that *fipA* may be expressed as part of an operon. To address this possibility, Northern blots were carried out with probes specific for ORF1 and *fipA* (see Materials and Methods). As shown in Fig. 4, the *fipA*-specific probe hybridizes to an mRNA of 1.4 kb, consistent with the size necessary to encode a 42.5-kDa protein. No transcript was detected with the upstream ORF probe.

Analysis of the expressed recombinant peptide. To confirm that the protein expressed by clone 13A′ was indeed a component of FIP, a modification of the immunoblot procedure was used to produce a monospecific serum against the immobilized and partially purified recombinant 44-kDa protein (12). As shown in Fig. 5, the antibodies that were eluted from the immobilized peptide expressed by clone 13A′ also reacted with the corresponding 44-kDa peptide derived from the native FIP. Of particular interest was the observation that this monospecific serum failed to react with the 48-kDa peptide. This suggests that these two peptides are immunologically distinct and are most probably products of different genes.

To determine if the recombinant 44-kDa protein, designated rFipA, possesses immunomodulatory activity, we first partially purified the peptide by following a protocol similar to that used for native FIP (see Materials and Methods). As shown in Fig. 5A (lane 2), significant enrichment of this peptide was

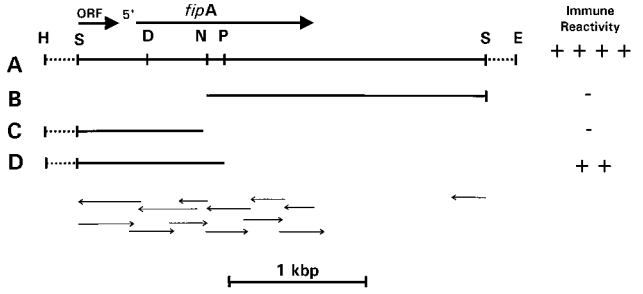

FIG. 2. Restriction map and DNA sequencing strategy of the *fipA* gene contained in clone 13A′. The location and orientation of the *fipA* gene are shown by the arrow. Restriction enzyme abbreviations are as follows: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; S, *Sau*3AI; P, *Pvu*II; N, *Nde*I; D, *Dra*I. Only the terminal *Sau*3A sites are shown. Also shown are the relative immunoreactivities of clone 13A′ and clones containing the 2.1-kbp (B) and 0.9-kbp (C) *Hin*dIII-*Nde*I fragments as well as the 1.0-kbp *Hin*dIII-*Pvu*II fragment (D). Broken lines represent vector DNA. The intensity of the immunoreactivity of each clone was graded from 1+ (weak reaction) to 2 to 3+ (moderate reaction) to 4+ (strong reaction); 0 indicates no detectable reaction. Restriction fragments were sequenced with the Sequenase version 2.0 kit, using either a universal primer or specific oligonucleotides (→).

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                                                                    60
      GATCAATGTTAAAAGCTAGAAAAGGTTCTATTATTACTCTATCATCAGTTGTTGGATTAC
        S  M  L  K  A  R  K  G  S  N  N  T  L  S  S  V  V  G  L  H
                                                                   120
      ATGGAAATGCTGGGCAAACAAATTATGCAGCAACAAAAGGTGGAGTAGTAGCAATGTCTA
        G  N  A  G  Q  Q  I  Y  A  A  T  K  G  G  V  V  A  M  S  K
                                                                   180
      AAACTTGGGCAAAAGAATTTGGTGGAAGAAATGTAAGAGCTAACTGTGTTGCTCCTGGAT
        T  W  A  K  E  F  G  G  R  N  V  R  A  N  C  V  A  P  G  F
                                                                   240
      TTATTCAAACACCTATGACTGATGTACTATCAGAAGATACAATAAAAGGAATGCTAGATG
        I  Q  T  P  M  T  D  V  L  S  E  D  T  I  K  G  M  L  D  A
                        -35                         -10
                                                                   300
      CAACTCCTCTTGGAAGACTTGGACAAGTTGAAGATATAGCAAATGGTGTATTATTTTTAG
        T  P  L  G  R  L  G  Q  V  E  D  I  A  N  G  V  L  F  L  A
                     -35                 -10
                                                                   360
      CAAGTGATGAGTCTTCATTTATAACTGGGGAAGTAATATCTGTATCTGGTGGTTTAATGC
        S  D  E  S  S  F  I  T  G  E  V  I  S  V  S  G  G  L  M  L
                                                                   420
      TTTAATTTCTTAGTTTGCAATTTTATTTTAGTATAACATTTTAAGGAGGAAGATATGAGT
                                                     M  S
                                                                   480
      AAAGTTTATGTAGTAGCAGCGAAAAGATCAGCTATTGGGAGTTTTTTAGGTAGTTTAGCA
  3   K  V  Y  V  V  A  A  K  R  S  A  I  G  S  F  L  G  S  L  A
                                                                   540
      CCTTTAAAACCTGGGGAATTAGGAGCTCAAATAGTAAAAAATATTCTTGAAGAAACAAAG
 23   P  L  K  P  G  E  L  G  A  Q  I  V  K  N  I  L  E  E  T  K
                                                                   600
      GTAGATCCAGCTAATATTGATGAAGTTATAGTTGGAAATGTTTTAAGTGCAGGACAAGCT
 43   V  D  P  A  N  I  D  E  V  I  V  G  N  V  L  S  A  G  Q  A
                                                                   660
      CAAGGTGTTGGAAGACAAGTTTCTATAAAAGCAGGAATACCTTATGAAGTTCCGGCTTAT
 63   Q  G  V  G  R  Q  V  S  I  K  A  G  I  P  Y  E  V  P  A  Y
                                                                   720
      TCTGTAAACATAATTTGTGGAAGTGGAATGAAATCAATTATAACAGCTTTCTCTAACATC
 83   S  V  N  I  I  C  G  S  G  M  K  S  I  I  T  A  F  S  N  I
                                                                   780
      AAAGCAGGCGAAGCAAATTTAGTAATAGCTGGTGGAACAGAATCTATGTCTGGTGCAGGA
103   K  A  G  E  A  N  L  V  I  A  G  G  T  E  S  M  S  G  A  G
                                                                   840
      TTTATTTTACCTGGTGCAATAAGAGGTGGACATAAGATGGCTGATCTTACTATGAAAGAT
123   F  I  L  P  G  A  I  R  G  G  H  K  M  A  D  L  T  M  K  D
                                                                   900
      CACATGATATTAGATGCTTTAACAGATGCTTATCATAATATTCATATGGGAATTACTGCT
143   H  M  I  L  D  A  L  T  D  A  Y  H  N  I  H  M  G  I  T  A
                                                                   960
      GAAAATATAGCAGAAAAATATGGAATAACAAGAGAAGAACAAGATGATTTTGCATTAGAA
163   E  N  I  A  E  K  Y  G  I  T  R  E  E  Q  D  D  F  A  L  E
                                                                  1020
      TCTCAAAAGAAAGCTATTGCAGCTGTTGATTCTGGAAAATTCAAAGATGAAATAGTTCCA
183   S  Q  K  K  A  I  A  A  V  D  S  G  K  F  K  D  E  I  V  P
                                                                  1080
      GTTGTTATTCCTAACAAAAAAGGAGATATTACATTTGATACAGATGAATATCCAAACAGA
203   V  V  I  P  N  K  K  G  D  I  T  F  D  T  D  E  Y  P  N  R
                                                                  1140
      AAAACAGATGCAGAAAAATTAGCTAAATTAAAACCTGCTTTCAAAAAAGATGGATCAGTT
223   K  T  D  A  E  K  L  A  K  L  K  P  A  F  K  K  D  G  S  V
                                                                  1200
      ACAGCAGGTAATGCTTCTGGACTTAATGATGGAGCATCATTTGTAATGCTTGCATCTGAA
243   T  A  G  N  A  S  G  L  N  D  G  A  S  F  V  M  L  A  S  E
                                                                  1260
      GAAGCAGTTAAGAAATATAATTTAAAACCATTAGTTGAAATTATTTCAACAGGTACTGGC
263   E  A  V  K  K  Y  N  L  K  P  L  V  E  I  I  S  T  G  T  G
                                                                  1320
      GGAGTAGATCCTTTAATAATGGGTATGGGACCAGTTCCAGCAATAAGAAAAGCATTTAAT
283   G  V  D  P  L  I  M  G  M  G  P  V  P  A  I  R  K  A  F  N
                                                                  1380
      AAAACAGATTTAAAATTAAAAGATATGGAATTAATTGAATTGAATGAAGCATTCGCTGCT
303   K  T  D  L  K  L  K  D  M  E  L  I  E  L  N  E  A  F  A  A
                                                                  1440
      GAATCATTAGGTGTTATAAAAGAACTTTGTAAAGAACATGGCGTAACTCCTGAATGGATT
323   E  S  L  G  V  I  K  E  L  C  K  E  H  G  V  T  P  E  W  I
                                                                  1500
      AAAGAAAGAACAAATGTAAATGGTGGAGCAATAGCATTAGGACATCCAGTTGGAGCTTCT
343   K  E  R  T  N  V  N  G  G  A  I  A  L  G  H  P  V  G  A  S
                                                                  1560
      GGAAACAGAATAACTGTTACATTAATCCATGAAATGAAGAAAAGAGGAGTAGAATATGGT
363   G  N  R  I  T  V  T  L  I  H  E  M  K  K  R  G  V  E  Y  G
                                                                  1620
      TTAGCTTCTCTATGTATAGGTGGAGGAATGGGAACTGCTCTTATTCTTAAAAATGTAAAA
383   L  A  S  L  C  I  G  G  G  M  G  T  A  L  I  L  K  N  V  K
                                                                  1680
      TAGTTAAAAATAACTAATGTACTGACCTTAAAAAGTCAAATATATTTAATCTAACCTATC
                                                                  1740
      AACAAGGATTGACTTCTGTAAAAAGCAGAGGTTAATCCTTTTTTATGTAAAAGAAAAGGT
                                                                  1800
      ATCCAATTTCTTGAATACCTAATCTTATATTTTTAAAATCTAGTTTTTAGAATGTATATC
                                                                  1860
      CACCTTTGATTCCAATTTTTACATTGTTTTCTTTTGAGTTAGTTATATATGTGCCTTCAA

      GTCCATAAGTATATCAACA
```

FIG. 3. Nucleotide and encoded amino acid sequences of the *fipA* gene. The numbers on the left count the encoded amino acids of the *fipA* gene; the numbers on the right count the nucleotides consecutively.

achieved compared with the crude lysate (Fig. 1, lane 2). The partially purified preparation of recombinant FIP was capable of carrying out functions similar to those already demonstrated for the native FIP. We first determined that rFipA suppressed

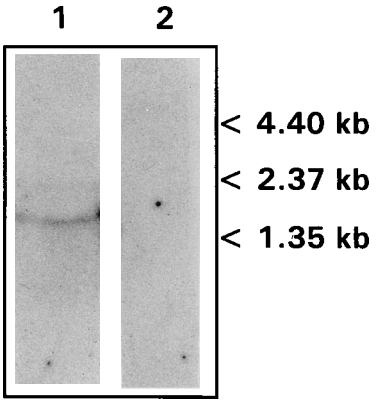

FIG. 4. Northern analysis of *F. nucleatum* FDC 364 RNA with probes specific for the *fipA* gene (lane 1) and the upstream ORF (lane 2) contained in pFipA. The relative positions of RNA size standards are shown on the right.

the proliferative response of human T cells to PHA in a dose-dependent manner (Fig. 6). The recombinant peptide was almost as potent as that of the native protein; the doses required to cause a 50% reduction in [^{3}H]thymidine incorporation were 1.6 and 0.2 μg/ml, respectively. Since suppression of cell proliferation, by itself, cannot be considered a unique feature of FIP, we assessed the effects of rFipA on other aspects of cell activation. For instance, cell cycle analysis indicated that, like cells exposed to native FIP, the rFipA-treated cells remained in the G_0/G_1 phase of the cell cycle (Fig. 7); it should be noted that the methods used did not discriminate between G_0 and G_1. Therefore, it was important to also demonstrate that the rFipA-treated cells had actually entered and been arrested in the G_1 phase of the cell cycle; this was achieved by assessing expression of the activation markers CD69 and CD25. As shown in Fig. 8, rFipA- and native FIP-treated cells expressed both CD69 and CD25 to the same degree as those cells exposed to mitogen alone, suggesting that the cells had indeed entered the G_1 phase of the cell cycle. Finally, as reported for the native FIP, T cells exposed to rFipA also failed to express the proliferating-cell nuclear antigen (PCNA ([data not

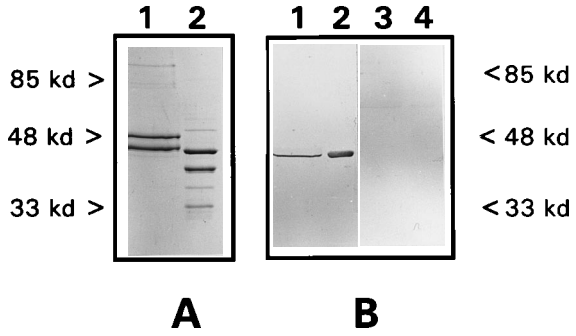

FIG. 5. Demonstration that clone 13A′ contains epitopes present on native FIP. Preparative immunoblots were prepared from cell lysates following SDS-PAGE and were used to isolate rabbit monospecific antibodies bound to the immobilized rFipA. Bound antibodies were eluted with 3 M KSCN, dialyzed, and used to probe immunoblots of native FIP. (A) SDS-PAGE analysis of purified native FIP (lane 1) and the partially purified rFipA (lane 2); gels were silver stained. (B) Immunoblot analysis of the eluted antibodies. Lanes: 1, native FIP probed with the monospecific antibodies to rFipA; 2, recombinant 44-kDa protein probed with monospecific antibodies to rFipA; 3 and 4, native FIP and the recombinant 44-kDa protein, respectively. Lanes 3 and 4 were probed with antibodies eluted from regions of the preparative immunoblot that did not contain the 44-kDa peptide.

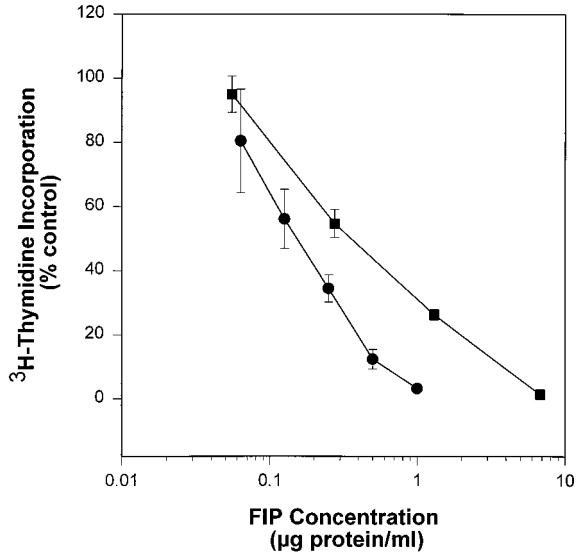

FIG. 6. Assessment of partially purified rFipA protein for biologic activity. HPBMC were incubated with various amounts of rFipA (squares) or purified native FIP (circles) for 30 min, and then an optimal mitogenic dose of PHA was added. [^{3}H]thymidine incorporation was measured after incubation for 4 days. The results are plotted as the percentage of [^{3}H]thymidine incorporation in control cultures receiving mitogen alone. Each point represents the mean ± standard error of a representative experiment performed in quadruplicate culture. Incorporation into control cultures exposed to medium alone averaged 167 cpm, and incorporation into cultures exposed to PHA alone averaged 17,232 cpm.

shown]). Collectively, these observations are consistent with the notion that rFipA causes a cell cycle arrest in the middle of the G_1 phase as previously demonstrated for native FIP (21).

DISCUSSION

F. nucleatum has been shown to produce an immunoinhibitory protein (FIP) capable of suppressing human B- and T-cell responsiveness. FIP is composed of two subunits, of 44 and 48 kDa. In this report, we have clearly identified a recombinant clone (13A′) that expresses the 44-kDa subunit, designated rFipA, of the *F. nucleatum* immunosuppressive factor. The expressed protein was not only recognized by monospecific sera to FIP but was also capable of inhibiting human T-cell activation in a manner consistent with that of the native FIP.

The cloned 3.1-kbp insert contains the entire *fipA* gene and two other potential ORFs, one upstream (ORF1) of *fipA* and transcribed from the same strand as *fipA* and the second downstream of *fipA* and transcribed from the opposite strand. Sequencing of the insert showed that a short intergenic region exists between ORF1 and *fipA* and that no rho-independent transcription sequence was present. The relatively short intergenic domain between ORF1 and *fipA*, as well as the lack of a rho-independent transcriptional sequence, raises the possibility that *fipA* is expressed as part of an operon. However, Northern blots with probes specific for ORF1 and *fipA* (see Materials and Methods) showed that the *fipA* transcript was 1.4 kb in size, consistent with the size necessary to encode a 42.5-kDa protein but not sufficient to encode ORF1. This suggests that *fipA* is transcribed only from its own promoter. Analysis of the sequence upstream of the *fipA* translational start codon identified two potential sets of −10 and −35 promoter elements on the basis of their similarity to the consensus *E. coli* −10 and −35 sequences. It is not known at present if either or both of these regions are functional in vivo. No transcript was detected with the ORF1 probe, suggesting that this gene is transcribed at very low levels or not at all under the culture conditions used.

A comparison of the *fipA* sequence to the GenBank database indicated that *fipA* is similar to a family of genes encoding enzymes involved in fermentation pathways in anaerobic microorganisms. The gene with greatest similarity to *fipA* was the acetyl coenzyme A *C*-acetyltransferase (thiolase) gene from *Clostridium acetobutylicum*; these genes shared 61.9% identity in a 1,072-bp overlap. Furthermore, the *C. acetobutylicum* gene encodes a protein of 392 amino acids which is similar in size to FipA (25). It is not known if FipA is capable of thiolase activity or if the *C. acetobutylicum* protein is able to modulate immune responses, but it is tempting to speculate that these enzymatic activities may be related to the mechanism by which FIP inhibits lymphocyte activity. Clearly, the relationship, if any, between these two proteins will be the focus of future investigation.

The deduced amino acid composition of FipA is not unusual. It possesses relatively equal numbers of positively and negatively charged residues. If all the charged residues were accessible to titration, the calculated pI was 7.62; this is consistent with previous observations that FipA elutes from anion-exchange columns (21) (Mono Q) at low salt concentrations. FipA also contains relatively equal numbers of hydrophilic and hydrophobic residues, resulting in a net hydrophobicity of −0.027 by the procedure of Kyte and Doolittle (11). However, the distribution of these hydrophobic residues is not uniform. A distinct hydrophobic region is evident near the N terminus, a hydrophilic region exists near the center of the polypeptide

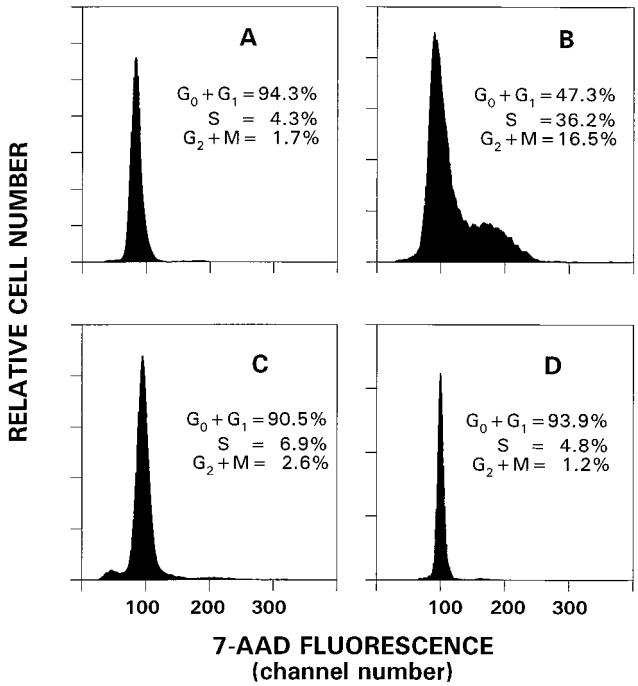

FIG. 7. Effect of rFipA on cell cycle progression. HPBMC were incubated with medium (A), PHA (B), PHA and 1 µg of rFipA per ml (C), or 0.5 µg of native FIP per ml (D) for 72 h. Cells were then stained with anti-CD3 conjugated to FITC and 7-AAD as described in Materials and Methods. Cell cycle analysis was performed by FACS after gating on $CD3^+$ cells. The abscissa is a linear display of 7-AAD fluorescence intensity, which corresponds to DNA content. Results are the mean of replicate cultures and are representative of three experiments.

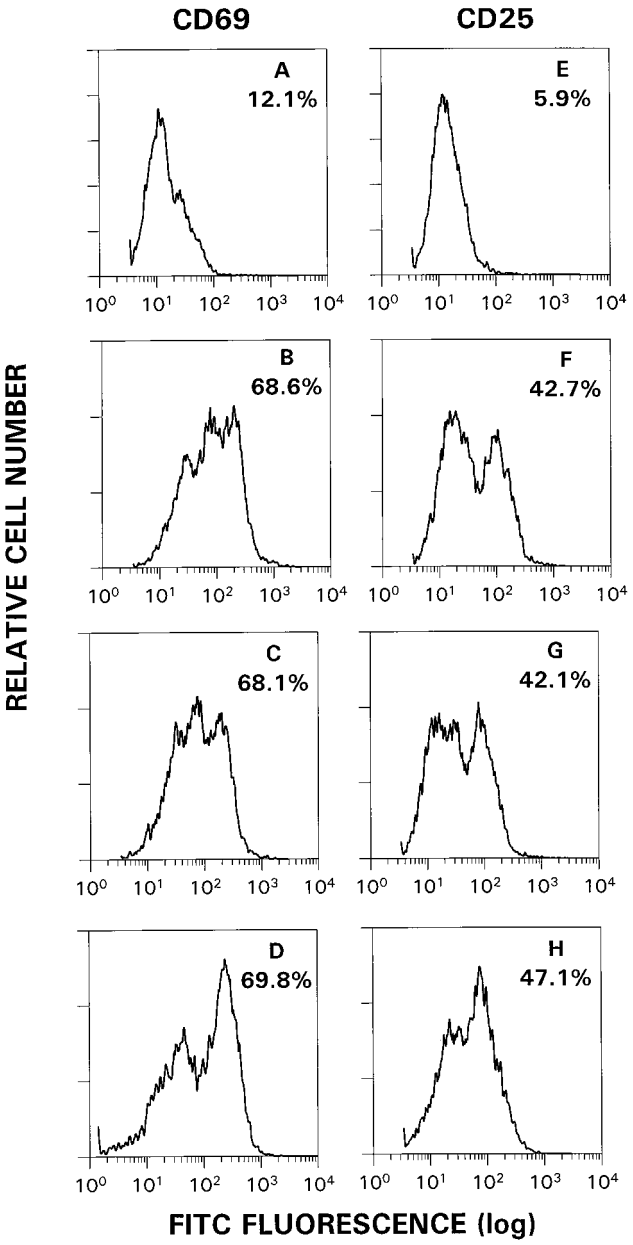

FIG. 8. Effect of rFipA on the expression of activation markers in T cells. T cells were incubated with medium (A and E), PHA (B and F), PHA and 1 μg of rFipA per ml (C and G), or PHA and 0.5 μg of native FIP per ml (D and H) for 24 h, and the cells were stained as described in Materials and Methods. Results are displayed for each marker, CD69 and CD25. The numbers in each panel represent the percentage of positive cells; analysis gates were set so that ≥98% of cells stained with isotypic control antibodies remained outside the positive region. Results are representative of three experiments; at least 10,000 cells were analyzed per sample.

chain, and alternating hydrophobic and hydrophilic patches occur in the last third of the protein.

Prediction of the secondary structure of FipA by the method of Chou and Fasman demonstrates a protein in which 54% of the residues are predicted to be in the α-helical conformation and 23% are in β turns (3, 16). The presence of the large number of turns suggests that the protein is capable of folding into a compact three-dimensional structure. The most notable features of this model are three hydrophilic turns (residues 170 to 178, 208 to 228, and 341 to 348), which are likely to be found exposed on the surface and may represent antigenic sites, and a long helical segment (residues 296 to 334). It is noteworthy that deletion analysis with *Pvu*II resulted in expression of a truncated peptide (25 kDa) that lacked two of these putative antigenic sites; this peptide exhibited a marked loss of immunoreactivity. The helical segment occurs in the amphipathic C-terminal domain. Helical-wheel analysis of this sequence suggests the occurrence of discrete hydrophobic patches along the helical axis (19). This impression is reinforced by the helical hydrophobic-moment calculation of Eisenberg et al. (6) and the discrete Fourier transform method of Kuhn and Leigh (10), which reveal peaks of recurrent hydrophobic residues. Amphipathic segments in proteins are thought to be responsible for protein-protein or protein-lipid interactions (7). Of interest here is that native FIP appears to consist of two subunits. It is possible that the interactions of these two peptides are initiated through this domain. Experiments to address this possibility are under way.

It is noteworthy that not only did the pFipA express a 44-kDa protein that was immunoreactive but also it was biologically active. Partially purified preparations of the recombinant protein inhibited human T-cell proliferation in a dose-dependent fashion. Cell cycle analysis indicates that these cells remained in the G_0/G_1 phase of the cell cycle. However, T cells exposed to FipA and mitogen-expressed activation markers, CD69 and CD25, to the same level as that observed in control cultures exposed to mitogen alone. These results indicate that the rFipA-treated cells did indeed proceed through the early stages of cell activation and enter the G_1 phase of the cell cycle. Like the native FIP, the earliest detectable lesion in rFipA-treated cells was impaired expression of proliferating-cell nuclear antigen; collectively, these results suggest that rFipA inhibits T-cell activation by causing a block in the G_1 phase of the cell cycle. These results are of particular interest because they indicate that the rFipA subunit peptide is sufficient to carry out the biologic function associated with the native FIP. It should be noted, however, that the immunosuppressive ability exhibited by rFipA was less potent than that observed with native FIP. This could be the result of differences in purity or may reflect the absence of the 48-kDa peptide.

The relationship of the 44- and 48-kDa peptides is not clear. We have not been able to successfully separate and individually purify the two subunits of the native FIP. One explanation may be that the two subunits copurify because they are products of a single gene and the difference in molecular masses might simply reflect a processing event. However, antibodies specific to the rFipA recognized only the native 44-kDa subunit and failed to react with the 48-kDa peptide. Therefore, it is more likely that the two FIP subunits are products of different genes. A surprising result was that rFipA possessed potent immunomodulatory activity in the absence of the 48-kDa subunit. However, the activity was lower than that of the native peptide control, suggesting that the 48-kDa subunit may be necessary for optimal activity. One possibility is that the 48-kDa polypeptide also possesses immunomodulatory activity on its own. It is also possible that it plays a structural role that is required by FipA for optimal activity. Thus, to fully understand the mechanism by which FIP acts, it will be necessary to delineate the function of the 48-kDa FIP subunit. We anticipate that the class I clones may contain a portion of the gene that encodes the 48-kDa protein; further analysis of these clones may help to clarify the relationship between these two components of FIP.

As already mentioned, *F. nucleatum* has been implicated in the pathogenesis of several diseases including periodontal dis-

orders. Of particular importance to our study are several reports that some patients with severe periodontitis have significantly altered immune system function (1, 4, 9, 13, 30). The immunologic mechanisms involved in the pathogenesis of *F. nucleatum* infections in general and periodontal disease in particular are not clearly defined. Enhanced susceptibility to infectious disease is a commonly acknowledged complication in patients with overt immunodeficiency. Less widely appreciated, however, are observations of immunologic dysfunction as a sequela to microbial infection in an otherwise healthy person. In this regard, it is becoming increasingly more apparent that microbial pathogens may have adverse effects on the immune system. These may include toxicity and interference with normal immunoregulatory processes, leading to anergy or failure of the immune system. There are several mechanisms by which microorganisms may evade the bactericidal effects of the host immune response. For instance, some organisms are capable of suppressing the immune system response through various products including toxins, enzymes, cell wall components, and metabolites (reviewed in references 20 and 27). In this context, the immunomodulatory protein produced by *F. nucleatum* is of considerable interest since it represents a potential virulence factor in infections caused by this organism. Identification of the underlying mechanism(s) by which FIP acts will not only facilitate an understanding of abnormal immune system function associated with *F. nucleatum* infections but will also contribute to a better appreciation of normal lymphocyte activation. The molecular cloning of the *fipA* gene represents an important first step toward these goals.

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