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Myosin-Cross-Reactive Epitope of *Shigella flexneri* Invasion Plasmid Antigen B

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IpaB, invasion plasmid antigen B, of *Shigella flexneri* is a 62-kDa protein required for invasion of intestinal epithelial cells. IpaB is also one of several major protein antigens recognized by the humoral immune systems of most humans and monkeys after infection with shigellae. Computer analysis of the deduced IpaB amino acid sequence indicates that an alpha-helical structure is likely through much of the molecule. Homology searches with protein data banks show that one alpha-helical domain between amino acid residues 95 and 181 has a moderate level of identity with myosin and streptococcal M protein. By using a monoclonal antibody (2F1) which recognizes an epitope in the amino-terminal third of the IpaB protein, it was possible to demonstrate a cross-reactive epitope(s) on skeletal muscle myosin. Epitope mapping localized the 2F1 epitope to three noncontiguous regions of the IpaB protein within the alpha-helical domain that contains homology with myosin. Antibodies produced in rabbits immunized with synthetic peptides from one of the 2F1 epitope regions (residues 99 to 110) of IpaB were capable of reacting with IpaB as well as myosin. Furthermore, sera from several monkeys previously infected with *S. flexneri* 2a contained antibodies to IpaB pep 101-116 (IpaB peptide 101-116) and also myosin. Sera from animals with antibodies against other IpaB peptides did not contain antibodies against myosin.

Shigellae are pathogenic microorganisms capable of invading and replicating freely within the cytoplasm of eukaryotic cells. The invasion process of *Shigella* species is a very complex, highly regulated event that involves several direct confrontations of the pathogen with various host cell membranes and proteins. This interactive process suggests that the pathogen is using host cell structures to assist in various steps such as phagocytosis (entry), intracellular movement, and eventual escape from the host cell. The *ipa* genes (*ipaB*, *ipaC*, and *ipaD*) (7) and the *virG* (*icsA*) gene, all encoded by the large virulence plasmid of *Shigella flexneri*, are thought to be critical elements in the invasive process (2, 3, 17). These genes and their corresponding proteins are unique to *Shigella* spp. and enteroinvasive *Escherichia coli* in that DNA homology or antigenic cross-reactivity with other invasive enteric pathogens have not been found (19, 35).

The IpaB protein is a major antigen of *Shigella* species that is almost always recognized by convalescent sera from monkeys or humans previously infected with shigellae (22). The IpaB protein, with a molecular size of 62 kDa, is soluble in aqueous buffers and contains long stretches of hydrophilic residues (34). By using monoclonal antibody (MAb) probes, it is possible to detect IpaB on the surface of invasive shigellae, yet this protein is not readily found in purified outer membranes of virulent shigellae (19, 21), suggesting that IpaB is weakly bound to the surface somewhat like a peripheral membrane protein. Recently, Mills et al. (19) characterized several epitopes of IpaB with MAbs and noted that one MAb (2F1), recognizing an epitope in the amino-terminal third of the molecule, was capable of reducing the plaque-forming capacity of shigellae, suggesting that IpaB was actively involved in the invasion phenotype. Because plaque formation is the net result of attachment, phagocytosis, intracellular replication, and subsequent intercellular spreading (24), it was not possible to determine the specific event inhibited by MAb 2F1. Another protein (VirG) is actively involved in both intra- and intercellular spreading and is associated with the actin accumulation which occurs during the spreading of the shigellae (3, 17, 25). However, the entry process and subsequent intracellular movement along stress fibers can occur in shigellae lacking a functional *virG* (*icsA*) gene (33). This latter type of movement (called organellelike movement) is thought to be involved in the migration of the bacteria toward the nucleus early after infection (33). The additional virulence components required to stimulate the entry process and responsible for the organellelike movement have not been defined, although the Ipa proteins are candidates.

In this study, we demonstrate that MAb 2F1 recognizes a common antigenic structure in IpaB and myosin. The cross-reactive epitope was in the region exhibiting a moderate level of amino acid homology between these two proteins. By using synthetic peptides from the 2F1 epitope region of IpaB, it was possible to generate antibodies against myosin in rabbits. In addition, monkeys with high serum antibody levels against IpaB peptide (pep) 101-116 were also shown to contain antibodies against myosin.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The invasive *S. flexneri* serotype 5 strain M90T-W and its noninvasive, virulence-plasmid-free, isogenic derivative M90T-55 are from the Walter Reed collection. Bacterial cultures were routinely grown in Penassay broth (Difco Laboratories, Detroit, Mich.) at 37°C.

Purified proteins and other reagents. Rabbit, chicken, porcine, and bovine muscle myosin were purchased from Sigma Chemical Co. (St. Louis, Mo.). Myosin from rat, guinea pig, and mouse skeletal muscle were prepared as described by Perry (26). Myosin subfragments 1 and 2 (SF1 and SF2, respectively) were obtained from Sigma. Protein molecular size standards were obtained from GIBCO/BRL

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(Gaithersburg, Md.) and Diversified Biotech (Newton Centre, Mass.). Mouse ascitic fluid containing MAbs 2F1 and 1H4 were kindly provided by J. Mills.

Immunoblotting procedure. *Shigella* polypeptides of whole-cell sodium dodecyl sulfate lysates (22), and preparations of purified myosin were separated on gels consisting of 9 or 13% acrylamide cross-linked with *N,N'*-diallyltartardiamide in a discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis system using Laemmli buffers (14) and electroeluted onto nitrocellulose (Bio-Rad Laboratories, Richmond, Calif.), as previously described (6, 22). MAbs against the IpaB protein of *S. flexneri* have been previously described (19). The nitrocellulose blots were probed with antisera and developed with alkaline phosphatase-labeled protein A (Kirkegaard & Perry, Gaithersburg, Md.) by the method of Mills and coworkers (19).

Epitope scanning of IpaB with MAb 2F1. Overlapping octamer peptides derived from the IpaB amino acid sequence (34) were prepared essentially as originally described (11) but with some modifications (31). Peptide synthesis was carried out on prederivatized polyethylene pins (Cambridge Research Biochemicals, Wilmington, Del.) which were deprotected, washed, neutralized, washed, and amino acylated (using blocked 9-fluorenylmethoxycarbonyl amino acids) (Milligen, Bedford, Mass.) repeatedly until the octamers were completed. Next, the peptides were acetylated, deblocked, and washed once more. Prior to the first enzyme-linked immunosorbent assay (ELISA), the covalently bound peptides were subjected to three rounds of ultrasonic disruption. The octameric peptides were screened with MAb 2F1, which was previously described to recognize an epitope in the amino-terminal third of IpaB (19). The antibodies (final dilution, 1:200) were incubated with the immobilized peptides overnight. Next the pins were washed, probed with goat anti-mouse immunoglobulin G (IgG) (conjugated with alkaline phosphatase) and developed with the phosphatase substrate (*p*-nitrophenyl phosphate) as previously described (31). The conjugate was also incubated with the pins to determine the degree of nonspecific binding by the conjugate. The sequence selected was the overlapping region between the first 20 kDa of IpaB (which was previously shown to contain the 2F1 epitope) (19) and that region containing sequence homology with myosin.

Peptide synthesis and immunization of animals. Peptides of the IpaB protein were synthesized by using the deduced amino acid sequence (34). The synthetic peptides used in this study and their sequences (using the single-letter amino acid codes) are as follows: pep 88-100+C, QILGEKSL TALTNC; pep 93-106+C, KSLTALTNKITAWKC; pep 101-116+C, KITAWKSQQQARQQKNC; pep 110-122+C, QARQQKNLEFSDKC; and pep 115-130+C, KNLEFSD KINTLLSETC. A C-terminal cysteine residue was added to each peptide for coupling to carrier proteins. Peptides were synthesized using *tert*-butyloxycarbonyl chemistry. Peptides were purified by high-pressure liquid chromatography and subsequently conjugated to either preactivated maleimide-keyhole limpet hemocyanin (KLH) or bovine serum albumin (BSA), using the Immject (Pierce Chemical Co., Rockford, Ill.) kit. Rabbits (two rabbits per peptide conjugate) were immunized with 200 μg of the KLH-peptide conjugates on four occasions separated by 2-week rests. The first injection contained the conjugate mixed with complete Freund's adjuvant, the second injection contained the conjugate mixed with incomplete Freund's adjuvant, and the third and fourth booster injections contained the conjugate and no adjuvant. Protein concentrations were determined by the bicinchoninic acid assay (Pierce Chemical Co.) (4), with BSA as the standard.

Enzyme-linked immunoassay. IpaB peptide-BSA conjugates (diluted in 0.2 M carbonate buffer, pH 9.8) were added to polystyrene microtiter plates at a quantity of 1 μg per well and allowed to attach overnight at 4°C. After being blocked with casein, serial twofold dilutions of pre- and postimmunization sera from immunized rabbits were incubated with the peptide conjugates for 2 h. The plates were subsequently washed, probed with goat anti-rabbit IgG and IgM conjugated with alkaline phosphatase, and developed with *para*-nitrophenyl phosphate as previously described (19). In assays using monkey sera, the peptide-KLH conjugates were used as the antigen and the probe was goat anti-human IgG conjugated with alkaline phosphatase. Rabbit skeletal myosin (Sigma) was used at a concentration of 1 μg per well.

RESULTS

Sequence homology between IpaB, myosin, and streptococcal M protein. Amino acid sequence homology with proteins in the National Biomedical Research Foundation protein data base was determined by using the FastP algorithm (16). Initial searches indicated that the heavy chains of human myosin, rabbit skeletal muscle myosin, rat skeletal myosin, and streptococcal M protein had moderate homology scores. The sequence homology between IpaB and human heavy-chain myosin and the type 24 M protein is shown in Fig. 1. In the tail region of human myosin, there was 21.2% identity in a 297-amino-acid overlap with IpaB, while for M24 and IpaB, there was 20.4% identity in a 167-amino-acid overlap. The region of IpaB that contained sequences homologous to both myosin and the M protein was between amino acid residues 82 and 182. Comparisons of sequences from other species of myosin or different serotypes of streptococcal M proteins consistently demonstrated regions of homology within this region of the IpaB protein (data not shown).

The streptococcal M protein and the myosin tail are examples of alpha-helical coiled-coil structures (18, 36). Computer analysis of the IpaB amino acid sequence revealed that it also has a sequence which may form a helical structure (Fig. 2). One of the predicted helical regions of IpaB (between amino acid residues 85 and 220) overlapped the sequence homologous with myosin and the streptococcal M protein. Figure 3 shows the characteristic 7-mer (abcdefg) arrangement often found in alpha-helical structures (9, 18) as it applies to IpaB. For residues 95 through 164, the characteristic pattern is followed by IpaB, with hydrophobic residues at the first (a) and fourth (d) positions and basic or acidic amino acids usually found at residues e and g. At residues 170 and 173, proline residues are found which may disrupt or kink the alpha-helical structure.

MAb 2F1 recognizes rabbit skeletal myosin. Previously it was reported that MAb 2F1 recognizes the amino-terminal third of IpaB in that it reacted with a truncated IpaB product of about 14 kDa (19) which contains a significant amount of the region with homology to myosin. The 2F1 epitope is localized on the surfaces of invasive shigellae and may play a role in the invasion of eukaryotic host cells in that the plaque-forming capacity of shigellae is reduced if the organisms are pretreated with MAb 2F1 (19). Because of the moderate degree of amino acid homology, similar predicted alpha-helical structures, and recent reports suggesting that cytoskeletal proteins are involved in the intracellular move-

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41'                                                    LGMQLQKKIKELQA  Hum Myosin

 1" MHNVSTTTTGFPLAKILTSTELGDNTIQAANDAANKLFSLTIADLTANQNINTTNAHSTS  IpaB
                    ........ ::. .. .: . ..: :.. ...: ..
78*                       FNNKALKDHNDELTEELSNAKEKLRKNDKSLSEKASKIQ  M24

55' RIEELEEEIEAERTSRAKAEKLRSDLSRELEEIS-ERLEEAVGATSTQIEMKKKREAEF-  Hum Myosin
                 : ..: . .:: : . :.. ..: ..: . ..... ::
61" NILIPELKAPKSLNASSQLTLLIGNLIQILGEKSLTALTNKITAMKSQQQARQQKNLEFS  IpaB
.. .. .  :.:... ...   .. :.:: : . .:::.. .. ... .. . . . :
117* ELEARKADLEKALEGAMNFSTADSAKIKTL-EAEKAALAARKADLEKALEGAMNFSTADS  M24

113' QKMRRDLEEA-TL-QHE--ATAATL---RKKHADS-VAELGEQIDNLQRVKQKLE---KE  Hum Myosin
.:..  :.:. .:  ..  . ..   . :.::: . .:...:...:   .:.  :
121" DKINTLLSETEGL-TRD--YEKQIN---KLKNADSKIKDLENKINQIQTRLSNLDPESPE  IpaB
.::.:: .:...: .:.  :::..  ....:::::::.:.. .....: ..:. . .
176* AKIKTLEAEKAALEARQAELEKALEGAMNFSTADSKIKTLEAEKAALAARKADLEKALEG  M24

162' KSEMKMEIDDLASNMETVSKAKGNLEKMCRALEDQLSEKTKEEEQQINDLTALNQVEYSR  Hum Myosin
:.....: .:. ..... :... .:..  .....:.:.... :.:.....:..... .
175" KKKLSREEIQLTIKKDAAVKDRTLIEQRTLSIHSKLTDKSMQLEKEIDSFSAFSNTASAE  IpaB
 ...: ..
236* AMNFSTADSAKIKTLEAEKAALEARQAELEKALEGAMNFSTADSAKIKTLEAEKAALEAE  M24

222' QLDEKDTLETQLSRGKQAFTQQIEELKRQLEEEIKAKSALAHALQSSRH-DCDLLREQYE  Hum Myosin
:.:..... : :.. .: .. :. . .. ::..:.. :: ..::.::. . . ...:.
235" QLSTQQKSLTGLASVTQLMATFIQLVGKNNEESLKNDLALFQSLQESRKTEMERKSDEYA  IpaB
296* KADLEHQSQVLNANRQSLRRDLDASREAKKQLEAEHQKLEEQNKISEASR  M24

280' EEQEAKAELQRAMSKANSEVAQWRTKYETDAIQRTEELEEAKKKLAQRLQDAEEHVEAVN  Hum Myosin
.:   .:::.:: ... ...  :  .: : . ... . :   ..  :  ... :.:..
295" AEVRKAEELNRVMGCVGKILGALLTIVSVVAAAFSGGASLALAAVGLALMVTDAIVQAAT  Ipa B

340' AKCASLEKTKQRLQNEVEDLMIDVERTNAACAALDKKQTNFDKILAEWKQKCEETHAVLE  Hum Myosin
.. . .:.. ... ..: . .:..
355" GN-SFMEQALNPIMKAVIEPLIKLLSDAFTKMLEGLGVDSKKAKMIGSILGAIAGALVLV  IpaB

400' SFQKESRSLSTELFKIKNAYEESLDQLETLKRENKNLQQEISDLTEQIAEGGKRIHELEK  Hum Myosin
417" AAVVLVATVGKQAAAKLAENIGKIIGKTLTDLIPKFLKNFSSQLDDLITNAVARLNKFLG  IpaB

460' IKKQVEQESELQAALEEAEASLEHEEGKILRIQLEVNQVKSEVDRKIAEKDEEIDQMKRN  Hum Myosin
473" AAGDEVISKQIISTHLNQAVLLGESVNSATQAGGSVASAVFQNSASTNLADLTLSKYQVE  IpaB

520' HIRIEESMQSTLNAEIRSRNDAIR  Hum Myosin
533" QLSKYISEAIEKFGQLQEVIADLLASMSNSQANRTDVAKAILQQTTA  IpaB
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FIG. 1. Sequence homology between IpaB, myosin, and streptococcal M24 protein. A homology comparison was determined on sequences from a portion of the tail region of human (Hum) skeletal muscle myosin (28), the IpaB protein (34), and a portion of the streptococcal type M24 protein (20). The FastP algorithm was used to make the comparisons (16). Identical amino acids (:), conservative replacements (.), and insertions made for the purpose of optimizing alignment (–) are indicated. The numbers on the left side of the figure are the amino acid residue number for each of the three sequences, and the prime, double prime, and asterisk refer to the human myosin, IpaB, and M24 proteins, respectively.

ment of shigellae (3, 8, 33), we probed myosin proteins isolated from several different animal species with MAb 2F1. Figure 4 shows that MAb 2F1 recognizes myosin isolated from rabbit, porcine, bovine, rat, guinea pig, and mouse skeletal muscle but did not react with chicken myosin. Other

	a	b	c	d	e	f	g
95	L	T	A	L	T	N	K
102	I	T	A	W	K	S	Q
109	Q	Q	A	R	Q	Q	K
116	N	L	E	F	S	D	K
123	I	N	T	L	L	S	E
130	T	E	G	L	T	R	D
137	Y	E	K	Q	I	N	K
144	L	K	N	A	D	S	K
151	I	K	D	L	E	N	K
158	I	N	Q	I	Q	T	R
165	L	S	N	L	D	P	E
172	S	P	E	K	K	K	L
179	S	R	E	E	I	Q	L

FIG. 3. Seven-residue periodicity of IpaB sequence in the region homologous with myosin. Amino acid residues 95 through 185 are listed according to the heptapeptide repeat arrangement abcdefg noted for other alpha-helical proteins (18). Residues a and d are usually hydrophobic, and residues e and g are usually charged residues. The boxed areas contain the IpaB sequence determined to be reactive with MAb 2F1 by epitope-scanning experiments.

available IpaB MAbs (19) did not react with rabbit myosin (data not shown).

Localization of the 2F1 cross-reactive epitope to the tail region of myosin was accomplished by reacting MAb 2F1 with purified SF2 isolated from rabbit muscle myosin (Fig. 5). MAb 2F1 also reacted with a 120-kDa minor protein component of a SF1 preparation which is presumably heavy meromyosin, containing the globular head region as well as a substantial portion of the myosin tail region (36). MAb 2F1 did not react with the SF1 peptide band at about 90 kDa.

Localization of the IpaB epitope recognized by MAb 2F1. To further resolve the location of the myosin cross-reactive epitope, octamer peptides of IpaB were synthesized onto polypropylene pins according to the epitope-scanning procedure of Geysen et al. (11). The region of IpaB (amino acid residues 90 to 190) used in the epitope-scanning experiments was selected on the basis of (i) the size of the truncated IpaB 14-kDa molecule retaining 2F1 reactivity, and (ii) the region of amino acid sequence homology between both the IpaB protein and myosin and IpaB and the streptococcal M proteins. The MAb reacted with three different peptide regions of IpaB (pep 99-110, pep 136-148, and pep 170-181) (Fig. 6), indicating that a portion of the epitope is actually conserved in each of these sequences. It is interesting that within each epitope region there are several hydrophilic residues occupying the normally hydrophobic a and d positions of the 7-mer repeat structure. Control antibodies (in-

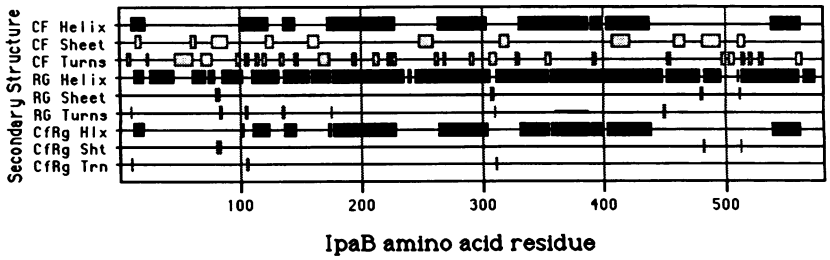

FIG. 2. Predicted secondary structure of IpaB. The predicted secondary structure of IpaB was determined by the Chou-Fasman (CF) method (7a) and the Robson-Garnier (RG) method (10a) using the MacVector sequence analysis program (International Biotechnologies, Inc., New Haven, Conn.). The third set of predictions is a consensus sequence of both the Chou-Fasman and Robson-Garnier (CfRg) determinations. Sequences compatible with helical structures (■) are indicated.

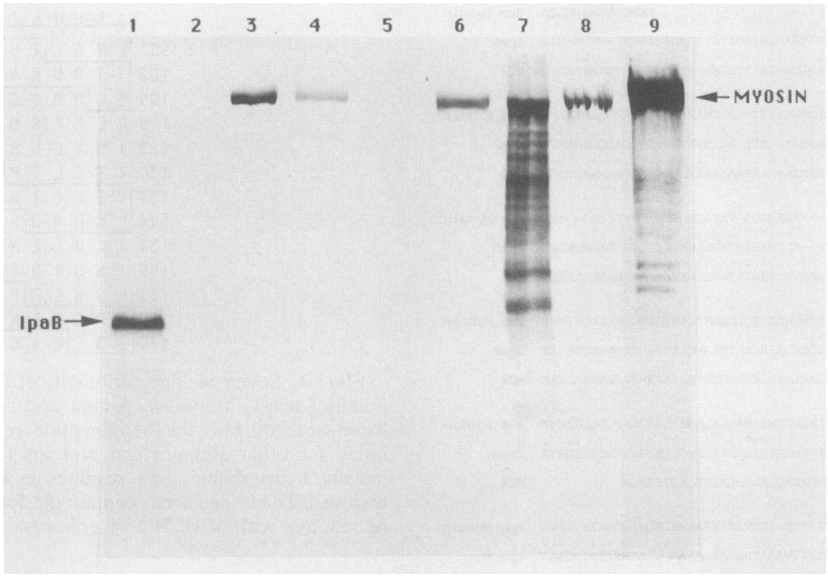

FIG. 4. Western blot analysis of different species of skeletal muscle myosin with the *S. flexneri* IpaB MAb. MAb 2F1 was reacted with different species of skeletal muscle myosin. The following antigens were analyzed in this blot: virulent *S. flexneri* 5 (lane 1), plasmid-free *S. flexneri* 5 (lane 2), and myosin from rabbit (lane 3), pig (lane 4), chicken (lane 5), cow (lane 6), rat (lane 7), guinea pig (lane 8), and mouse (lane 9) muscle. Only the chicken skeletal muscle myosin (lane 5) did not react with MAb 2F1. The bands beneath the rat (lane 7) and mouse (lane 9) myosin bands are degradation products still reactive with the MAb 2F1.

cluding MAb 1H4 as well as the goat anti-mouse conjugate) did not react with any of the synthetic peptides.

Synthetic peptides from 2F1 reactive region of IpaB elicit anti-IpaB and anti-myosin antibody response in rabbits. To determine whether the 2F1 reactive regions were capable of eliciting antibodies against IpaB and myosin, overlapping synthetic peptides representing amino acid residues 88 to 122 were used to immunize rabbits. Each synthetic peptide

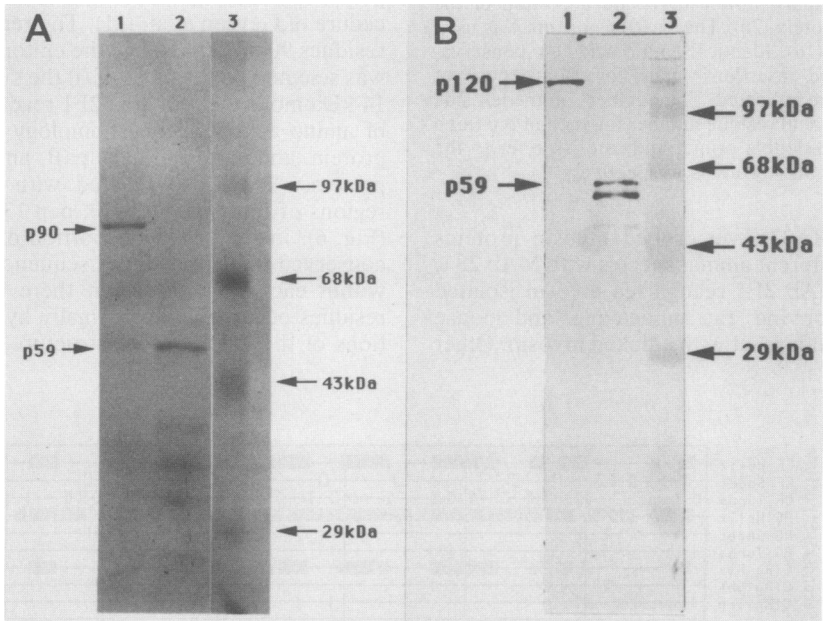

FIG. 5. Reactivity of myosin subfragments with MAb 2F1. Purified myosin SF1 (lane 1) and SF2 (lane 2) were electrophoresed and stained with Coomassie blue (A) and analyzed by Western blotting with MAb 2F1 (B). The major proteins in these purified preparations were SF1 at approximately 90 kDa (lane 1 of panel A) and SF2 at approximately 59 kDa (lane 2 of panel A). An additional fragment in the subfragment 2 preparation is light meromyosin. MAb 2F1 reacted with subfragment 2 (lane 2 of panel B), indicating that the cross-reactive epitope is in the tail region of myosin. The MAb also reacted with a 120-kDa protein antigen in the SF1 preparation (lane 1 of panel B), which is most likely a large contaminating peptide containing SF2 still linked to SF1 or light meromyosin. Lane 3 (both panels) contains prestained molecular mass standards.

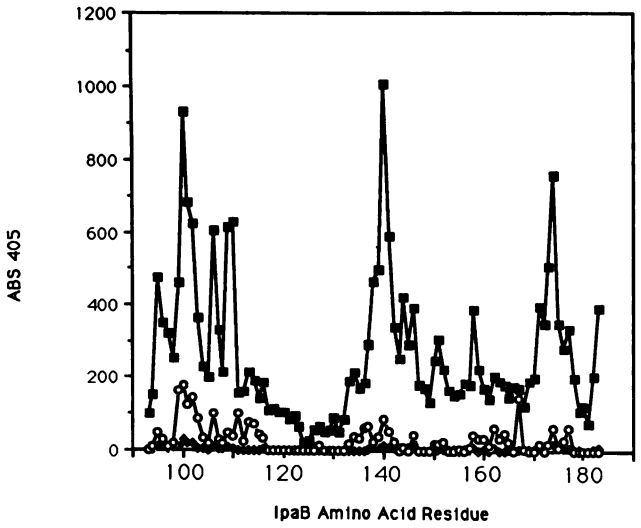

FIG. 6. Epitope scanning of the IpaB protein, using synthetic peptides. Synthetic octamer peptides of IpaB, scanning from amino acid residues 92 to 189, were reacted with either MAb 2F1 (■), anti-IpaB MAb 1H4 (○), or the goat anti-mouse conjugate (◆). MAb 2F1 reacted in three distinct regions defined by amino acids 98 to 109, 136 to 146, and 169 to 179 of the IpaB sequence. The antibodies bound to the synthetic peptides were detected with alkaline phosphatase-conjugated goat anti-mouse antisera. ABS 405, A_{405}.

produced an antibody response in rabbits against the homologous peptide as measured in an ELISA, with titers ranging from 1/200 to 1/6,400. To confirm sequence specificity, the portion of the immunizing peptide recognized by the rabbits was determined with the epitope-scanning system using overlapping octamers of the IpaB sequence (data not shown). Each rabbit responded against a unique sequence in a region contained within the immunizing peptide.

The rabbit sera produced against the IpaB peptides were also reacted against lysates of *S. flexneri* in Western blot (immunoblot) assays. Because the rabbits had high levels of preexisting antibodies reactive with antigens in the 60-kDa region of the gels in both noninvasive strains and invasive strains of shigellae, it was not possible to interpret the response against IpaB in the native organism (data not shown). However, by using a recombinant organism (S12) expressing a β-galactosidase–IpaB fusion protein (7), it was possible to demonstrate the presence of antibodies against IpaB epitopes (Fig. 7). Sera from rabbits immunized with IpaB peptides pep 88-100, pep 93-106, and pep 101-116 also produced antibodies which cross-reacted with myosin (Fig. 7).

Monkeys infected with shigellae respond to IpaB synthetic peptides from the myosin cross-reactive region. The serum antibody response against the IpaB peptides was also measured in monkeys challenged orally with virulent *S. flexneri*. Other studies have indicated that most humans and monkeys infected with shigellae produce a dominant serum antibody response against the Ipa proteins, as determined by Western blot analysis (22). Of the five monkeys used in this study, two (M19 and M23) demonstrated minimal levels of antibodies against IpaB peptides, while the sera from monkeys M25, M31, and M33 all contained antibodies against IpaB peptides (Table 1). The specificity of the anti-peptide response varied from animal to animal, in that high titers against pep 88-100 and pep 110-122 were found only in monkey M33, while all three monkeys had elevated titers against pep 93-106. In addition, the two monkeys (M25 and M31) with elevated titers (>1,600) against pep 101-116 also had demonstrable titers against myosin. Because M31 had preexisting antibodies against myosin and IpaB peptides, it could not be determined whether the response was due to a previous shigellae infection or some other cross-reacting antigen. Additional studies have indicated that of seven monkeys with serum antibodies reactive with myosin by Western

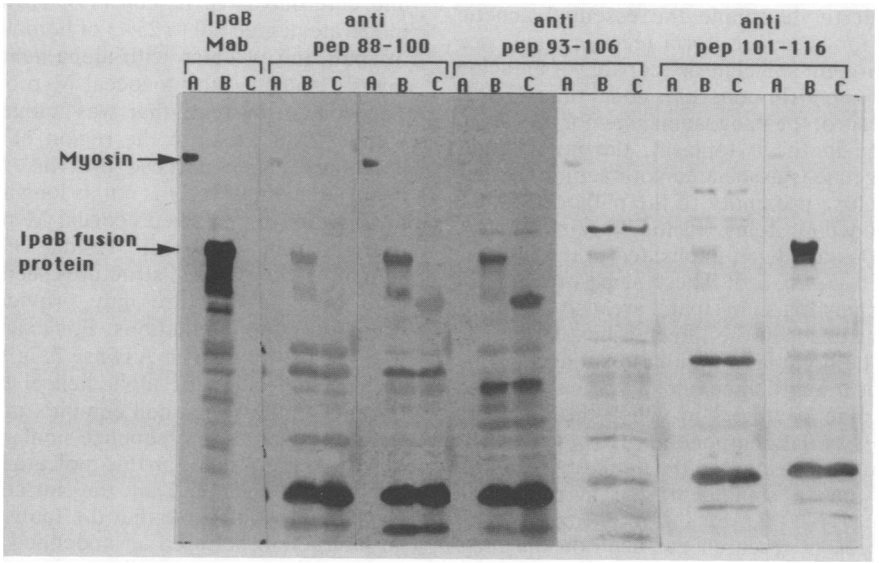

FIG. 7. Western blot analysis of myosin and IpaB with rabbit antisera generated against synthetic peptides of IpaB. Antisera from two rabbits immunized with the same peptide (indicated across the top of the figure) were incubated with the antigen strips within the brackets. MAb 2F1 (IpaB MAb) was used in the leftmost gel. The antigens used in these experiments were myosin (lanes A), a λgt11 recombinant (S12) expressing an IpaB–β-galactosidase fusion protein (6) (lanes B), and a λgt11 lysogen (lanes C). Rabbits immunized with pep 88-100, pep 93-106, and pep 101-116 produced antibodies against IpaB and myosin.

TABLE 1. Serum antibody response against IpaB peptides from myosin cross-reactive region[a]

Monkey and time of bleeding[b]	Antibody titer						
	IpaB peptide					KLH	Myosin
	pep 88-100	pep 93-106	pep 101-116	pep 110-122	pep 115-130		
M19							
Pre	<200	<200	<200	<200	<200	<200	<200
Post	<200	200	<200	<200	<200	<200	<200
M23							
Pre	400	800	400	400	400	400	<200
Post	400	400	400	200	400	400	<200
M25							
Pre	200	≥3,200	400	200	400	200	<200
Post	400	≥3,200	≥3,200	800	800	200	200
M31							
Pre	800	≥3,200	≥3,200	800	1,600	400	400
Post	800	≥3,200	1,600	800	1,600	200	400
M33							
Pre	12,800	1,600	800	6,400	6,400	<200	<200
Post	1,600	3,200	400	400	3,200	<200	<200
MAb 2F1	800	1,600	200	200	400	<200	≥204,800

[a] Synthetic IpaB peptides conjugated to KLH, KLH alone, and myosin, were used in an ELISA to determine the antibody response in monkeys after infection with *S. flexneri*. Serial twofold dilutions (starting at 1/200) of the monkey sera were used to determine the titer against each antigen. MAb 2F1 was also tested against each antigen preparation.

[b] Bleedings were taken before challenge (pre) and 4 weeks postchallenge (post).

blotting, six had detectable antibodies against pep 101-116 and all reacted with IpaB by Western blotting. In another group of four monkeys without myosin antibodies, only one had antibodies against pep 101-116 and all four had anti-IpaB antibodies by Western blotting (data not shown).

DISCUSSION

The ability of shigellae to actively invade and replicate freely within the cytoplasm of eukaryotic cells is a property shared by few bacteria, such as *Listeria monocytogenes* and the obligate intracellular rickettsiae (30, 32). The complex, multicomponent, invasion phenotype of *Shigella* species has made it very difficult to determine the essential genetic repertoire necessary for virulence (for a recent review, see reference 12). Only virulent shigellae are capable of inducing the initial phagocytic event (in nonprofessional phagocytes) and the subsequent lysis of the phagosomal membrane. Once the shigellae are free in the cytoplasm, the intracellular movement of the shigellae is associated with actin polymerization occurring in close proximity to the pathogen (3, 8). Several plasmid-encoded proteins, including VirG (IcsA), IpaB, IpaC, and IpaD, have been implicated in the interaction with the host cell (3, 25, 29). These same proteins, in addition to others such as IpaA and IpaH, are also the major protein antigens recognized by the infected host's immune system (7, 13, 22). The dominant immune response against proteins necessary for the virulence phenotype has resulted in many current vaccine strategies in which the invasion-related proteins are essential components. However, it has not been possible to demonstrate that the antibody response against the proteins has a significant role in protection against shigellae (10).

The focus of the present research has been on the IpaB protein. IpaB is a 62-kDa protein which is required for invasion, as determined by the inability of IpaB mutants to invade tissue culture cells (29). The accessibility of IpaB to antibodies in the whole-cell ELISA and the ability of MAb 2F1 to inhibit plaque formation by shigellae (19) suggest that IpaB is expressed on the surface of shigellae. Recent studies also indicate that IpaB is transported or released into the culture medium (1). Even so, the quantity of IpaB demonstrable in purified outer membranes is very low, relative to the quantity found in purified inner membranes or cytoplasmic extracts (1, 21). The release of IpaB from whole cells can be accomplished with water or physiological buffers, suggesting that it may be loosely associated with the outer membrane's external leaflet somewhat like a peripheral membrane protein (1, 21, 22).

Previously it was demonstrated with MAbs and by comparison of DNA sequences that IpaB is unique to *S. flexneri* and enteroinvasive *E. coli* (19). However, in this study a moderate degree (20 to 25%) of homology between a portion of IpaB and proteins with alpha-helical structures such as myosin and the streptococcal M protein has been shown. The domain of IpaB that was antigenically cross-reactive with myosin exists in the region of moderate amino acid homology between these proteins. The proposed alpha-helical domain of IpaB is not as long as the coiled-coil in the myosin tail or the streptococcal M protein, in that it only extends from amino acid residue 95 to 164. This size is similar to the repeated structures seen in some alpha helices (15). Such a structure may provide stability to certain protein-protein interactions. For example, although the active site of the myosin ATPase is in the head region of the myosin molecule, the alpha-helical tail structure provides stability to the coiled-coil complex of the tail (36). Because IpaB does not have a sequence similar to known ATPases, it may not be possible for this molecule to stimulate the actin polymerization noted in the intracellular movement of shigellae. It is possible that the IpaB molecule is promoting an interaction between an undefined host protein (possibly actin) and another shigella surface protein such as VirG. Definition of the function of IpaB will require isolation of the protein and its analysis in a model system. Alternatively, site-directed mutagenesis could be used to dissect the various functional regions of the IpaB protein.

The myosin cross-reactive epitope of IpaB was found to consist of at least three distinct, noncontiguous regions from amino acid residues 99 to 110, 136 to 148, and 170 to 181. This pattern of recognition by a MAb in the epitope-scanning experimental system suggests that the structure of the epitope is repeated even though the primary sequence is different. Similarily, the ability of the MAb to recognize other proteins (such as myosin) with similar secondary structures, but not identical primary sequences, suggests that a form of molecular mimicry exists between the IpaB epitope and the myosin epitopes recognized by the 2F1 MAb.

Other proteins, such as the M protein of *Streptococcus pyogenes*, contain epitopes which are cross-reactive with myosin (9). This immunological relationship is thought to be important in various autoimmune disorders (such as rheumatic heart disease or poststreptococcal glomerulonephritis) associated with streptococcus infection. Autoimmune disorders such as Reiter's syndrome and ankylosing spondylitis are associated with gram-negative organisms, including *Shigella*, *Klebsiella*, *Yersinia*, and other species (5). Several investigations have suggested that people carrying the HLA-B27 major histocompatibility antigen are more likely to generate autoreactivity associated with these gram-negative bacteria (5). In fact, several *Shigella* antigens, including OmpA and other smaller proteins, have cross-reactive epitopes with the HLA-B27 molecule (27, 37, 38). In the present study, we have shown that rabbits immunized with IpaB peptides pep 88-100, pep 93-106, and pep 101-116 are capable of producing an anti-myosin antibody response. In previously infected monkeys, one IpaB peptide in particular (pep 101-116) was associated with the presence of antibodies against myosin. In preliminary studies, we have found that several patients with various autoreactive diseases (such as Reiter's syndrome and ankylosing spondylitis) display an anti-myosin and anti-IpaB response that was most pronounced in one patient with the HLA-B27 marker (23). The cross-reactivity between IpaB and myosin warrants further investigation as a possible stimulus for the autoreactive diseases associated with shigellae, especially since IpaB is recognized by most individuals infected with shigellae (22). In addition to the antigenic relationship between IpaB and myosin, recent studies have demonstrated that the streptococcal M protein and an unidentified 70-kDa protein in *L. monocytogenes* also cross-react with the IpaB MAb (21). These additional cross-reactivities, especially with the streptococcal M protein, raise the possibility that a compound autoreactivity problem might occur in those individuals with histories of *S. pyogenes* infections and *Shigella* infections.

These studies have clearly demonstrated that the IpaB protein of *S. flexneri* has an epitope which is cross-reactive with myosin. Future studies will be directed toward determining the significance of IpaB in the invasion process, with specific attention to the myosin cross-reactive region and its possible interaction with host cell proteins. The role of IpaB in the anti-myosin response found in humans infected with shigellae will also be examined.

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