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Cloning and Sequence Analysis of a Gene Encoding a 67-Kilodalton Myosin-Cross-Reactive Antigen of *Streptococcus pyogenes* Reveals Its Similarity with Class II Major Histocompatibility Antigens

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The group A streptococcal sequela acute rheumatic fever (ARF) has been associated with immunological cross-reactivity between streptococcal and heart proteins. To identify *Streptococcus pyogenes* genes that encode a myosin cross-reactive antigen(s) recognized by ARF sera, a genomic library from an *emm* deletion strain (T28/51/4) was screened with a single ARF serum. A positively identified λEMBL3 clone (T.2.18) produced a protein which reacted with myosin-specific antibodies affinity purified from individual ARF sera. The recombinant protein was initially estimated to be 60 kDa in size by sodium dodecyl sulfate-polyacrylamide gel electrophoresis; however, upon sequence analysis it had a molecular mass equivalent to 67 kDa. Sera from patients with streptococcal infections, acute glomerulonephritis, and ARF were reactive with the recombinant 67-kDa protein. However, individual sera from healthy persons were negative or demonstrated low levels of reactivity with the 67-kDa antigen. The gene encoding the 67-kDa myosin-cross-reactive antigen was subcloned, and its nucleotide sequence was determined by using a combined strategy of DNA sequencing of the cloned gene and N-terminal amino acid sequencing of the protein expressed in *Escherichia coli*. The amino-terminal sequence deduced from the nucleotide sequence of an open reading frame was identical to that determined from the 67-kDa protein expressed in *E. coli*. The gene encoded 590 amino acids with a calculated molecular weight of 67,381. No cleavable signal peptide was detected with the 67-kDa protein expressed in *E. coli*. The deduced amino acid sequence of the 67-kDa protein did not exhibit significant similarity to any known streptococcal proteins. However, it was found to be 19% identical and 62% similar over 151 amino acid residues to the β chain of mouse major histocompatibility complex class II antigen (I-A^u). Similar degrees of homology to the β chains of other murine and human class II haplotypes were found. Mouse anti-IA sera reacted with the recombinant 67-kDa protein about five times more strongly than normal mouse sera in the enzyme-linked immunosorbent assay. Southern hybridization experiments using a probe for the gene encoding the 67-kDa protein showed that the gene was present and conserved among pathogenic groups A, C, and G of streptococci. These data suggest that the streptococcal protein, which is distinct from the M protein, may have structural features in common with the β chain of the class II antigens, as well as myosin, and may play an important role in the pathogenesis of streptococcal infections.

Streptococcus pyogenes is a common human pathogen that causes pharyngitis, and in some individuals group A streptococcal infection leads to acute rheumatic fever (ARF). Studies on immunological cross-reactivity between group A streptococci and host tissue targets such as myosin suggest an autoimmune mechanism for the pathogenesis of ARF (17, 18, 41, 42). Heart- or myosin-reactive antibodies which also react with group A streptococci are generated in ARF and may damage heart tissue directly or after exposure of cryptic autoantigens by other mechanisms. Therefore, characterization of streptococcal and heart antigens involved in this immunological cross-reactivity is important. Streptococcal components thought to be responsible for induction of heart-reactive antibodies are the M protein (9, 13, 14) and streptococcal membrane (12, 36). The antigens in the streptococcal membranes which react with heart- or myosin-reactive antibodies have not been defined.

Murine monoclonal antibodies produced against streptococcal membranes were found to react with undefined streptococcal membrane components, M protein, and myosin (7, 8, 11, 12, 19). Over the last decade, evidence that group A streptococcal M protein and myosin have epitopes in common has accumulated, (7–14, 19), and subsequent studies have demonstrated that epitopes in the M protein molecule react with myosin-specific antibodies (9, 13). The data are supported by the fact that the M protein has an alpha-helical coiled-coil structure very similar to that of myosin (21–23). Although streptococcal M protein, the major virulence factor of group A streptococci, has been extensively studied for its cross-reactivity with human tissues, few studies have addressed the role of other streptococcal proteins in cross-reactivity with host tissues or autoantigens such as myosin. Group A streptococcal molecules other than the M protein that share epitopes with myosin or heart tissue have been noted (3, 4, 36, 38, 41) but are not well characterized.

In our previous studies, a 60-kDa antigen that has epitopes in common with myosin, as demonstrated by its reactivity with myosin-specific antibodies, was identified in streptococcal extracts (3). This 60-kDa antigen was not the M protein as determined by its presence in an *emm* deletion strain (M negative). ARF sera, but not sera from healthy persons (normal sera), were highly reactive to the antigen found in streptococcal membranes (3). We have also characterized

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molecules in the streptococcal *emm* deletion strain that are functionally and immunologically similar to eucaryotic actin (4). These studies have characterized streptococcal proteins other than the M protein that have immunological and functional characteristics in common with eucaryotic contractile proteins.

We now present the results of cloning and sequence analysis of a streptococcal gene that encodes a 67-kDa myosin-cross-reactive antigen distinct from the M protein. It is shown that the gene for the antigen is present only in pathogenic streptococcal groups A, C, and G and that it does not have sequence identity to any known streptococcal proteins. However, significant similarity was observed between the streptococcal antigen and domain I of the β chain of class II major histocompatibility complex (MHC) antigens.

MATERIALS AND METHODS

Bacterial strains, phages, and growth conditions. An *emm* deletion (M-negative) strain (T28/51/4) of *S. pyogenes* (33) was obtained from the Rebecca Lancefield collection, The Rockefeller University, New York, N.Y., to construct a genomic library. Serotypes M5 and M24 of *S. pyogenes* were obtained from Edwin Beachey (Veterans Administration Medical Center, Memphis, Tenn.). Other streptococcal groups (B, C, and D and *S. mutans* Ingbritt) and *Staphylococcus aureus* 83-340 were obtained from Joseph Ferretti, Department of Microbiology and Immunology, University of Oklahoma Health Sciences Center, Oklahoma City, Okla. Streptococcal and staphylococcal cells were grown in Todd-Hewitt broth (Becton Dickinson & Co., Cockeysville, Md.) at 37°C. *Escherichia coli* LE392 (27) was grown exponentially at 37°C in Luria broth (L broth) supplemented with 0.2% maltose and 10 mM $MgSO_4$ with vigorous shaking and infected with recombinant phage clones at a low multiplicity of infection to prepare phage lysate. *E. coli* JM109 (39) cells were grown exponentially in L broth at 37°C and used for routine transformation with recombinant plasmids or with recombinant bacteriophage M13 DNA by the calcium chloride method (31). Transformants were selected on L broth agar containing ampicillin (50 μg/ml), 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal; 40 μg/ml), and isopropyl-β-D-thiogalactopyranoside (IPTG; 16 μg/ml) and grown overnight in 5 ml of L broth containing ampicillin at 50 μg/ml for plasmid isolation. Transparent phage plaques were picked and suspended in 1 ml of L broth. One-fifth of a resuspended plaque was mixed with exponentially growing JM109 cells in 3 ml of 2× YT medium and allowed to grow for about 5 h to prepare M13 DNA and single-stranded template DNA for DNA sequencing.

Human sera. The ARF and acute glomerulonephritis (AGN) sera and sera from patients with streptococcal infections used in our studies were from patients with above-normal anti-streptolysin O (ASO) titers (≥167 Todd units). The sera tested were taken from patients diagnosed during the acute stage of the disease and when the ASO titer was highly elevated. Sera were tested individually in this study and were not pooled. Although a single ARF serum was used for cloning of the antigen, it was similar in anti-myosin reactivity to other ARF sera tested subsequently against the antigen. All of the normal sera tested had ASO titers of ≤50 Todd units and were tested individually. In addition, the normal sera tested had very low anti-myosin antibody levels (titer, <100 when reacted in a Western immunoblot against myosin). Normal sera, ARF sera, and sera from patients with uncomplicated streptococcal infections have been studied previously for anti-myosin antibodies (10). A total of 20 ARF sera, 20 normal control sera, 19 AGN sera, and 10 sera from patients with uncomplicated streptococcal infections were reacted at a dilution of 1:2,000 with T.2.18 recombinant phage lysates in Western blots by using a method previously described (3).

Construction and immunological screening of the DNA library. A genomic library of *S. pyogenes* T28/51/4 was constructed by ligating partially digested *Sau*3A fragments of genomic DNA to λEMBL3 arms (Stratagene, La Jolla, Calif.) and by packaging the resulting recombinant DNA with a lambda packaging mixture (Amersham-Searle, Inc., Arlington Heights, Ill.). Recombinant phages were plated on *E. coli* LE392 and incubated overnight at 37°C. Plaques were lifted onto nitrocellulose filters and screened for production of ARF-associated antigens by using serum from a confirmed ARF patient. Approximately 4,500 recombinant plaques from the M(−) gene library were probed with this serum. Antibodies bound to plaque antigens were detected by using ^{125}I-labelled protein A. Selected clones were purified two or three times, until all of the plaques on a plate had positive signals by antibody screening. Purified clones were then analyzed for antigen production by Western blotting.

Isolation of phage DNA. To isolate phage DNA from phage lysate, cell debris was centrifuged (3,000 × *g* for 10 min) and the supernatant was mixed with an equal volume of phage dilution buffer (per liter, 5 g of NaCl, 1 g of $MgSO_4$, 50 ml of 1 M Tris-HCl [pH 7.5])–1 ml of 1 M $MgSO_4$–3.2 ml of DNase I solution (1 mg/ml) in phage dilution buffer–5 ml of 2% gelatin–20 ml of chloroform and then incubated for 15 min at 23°C. The phage was then precipitated by adding 20 ml of 5 M NaCl and 22 g of polyethylene glycol 6000 with incubation on ice for 2 h. The precipitated phage was centrifuged and resuspended in 3 ml of TM buffer (50 mM Tris [pH 7.4], 10 mM $MgSO_4$). The phage was extracted twice in an equal volume of chloroform, and then 30 μl of 0.5 M EDTA (pH 8.0) and 60 ml of 5 M NaCl were added to destabilize the phage particle. The phage DNA was then extracted with phenol and chloroform and ethanol precipitated. The prepared phage DNA was treated with DNase-free RNase A to remove bacterial RNA and again ethanol precipitated after phenol and chloroform extraction.

Plasmid subcloning. Phage DNA purified from a positive clone (λT.2.18) was used to map the cloned streptococcal DNA with restriction endonucleases. The streptococcal DNA was subcloned into pUC plasmids and subsequently into phage M13. Plasmid pKS40 (see Fig. 5) was constructed by substituting a 10-kb *Eco*RI-*Sal*I fragment of streptococcal DNA obtained from λT.2.18 DNA for the *Eco*RI-*Sal*I fragment in the multiple cloning sites of pUC19. Recombinant plasmid pKS40 was introduced into *E. coli*, and expression of the 67-kDa protein was analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and Western blot analysis.

Western blot analysis. Western blot analysis was performed as described previously (3), with slight modifications. *E. coli* cell extract was prepared from 1 ml of an overnight culture. Cells were collected in microcentrifuge tubes spun in a Biofuge A model no. 1217 (American Scientific Products) at 12,000 rpm for 5 min, resuspended in 1 ml of 5% SDS–cracking buffer, and diluted 10× in the same buffer. Phage lysates were mixed with equal volumes of 2× 5% SDS–cracking buffer. Proteins were solubilized by boiling for 10 min and then separated electrophoretically by SDS–10% PAGE with a constant current of 35 mA per gel. Proteins were electroblotted onto a nitrocellulose membrane (0.45-μm pore size; Bio-Rad, Richmond, Calif.) overnight at 80 mA. Blots were subsequently stained with amido black, and the unstained part was blocked

in 5% powdered milk–phosphate-buffered saline (PBS) for 1 h at 37°C before application of sera or antibodies. Myosin-specific antibodies were purified from the original ARF serum with a myosin affinity column prepared by attaching rabbit skeletal myosin (Sigma Chemical Co., St. Louis, Mo.) to glutaric dialdehyde-activated silicate carrier material (Boehringer Mannheim Inc., Indianapolis, Ind.) as described previously (10). Normal and ARF sera were applied at a 1:2,000 dilution, and the blots were incubated overnight at 4°C. After being washed with 2% Tween–PBS, an anti-human polyvalent immunoglobulin-peroxidase conjugate (Sigma) was diluted 1:100 in 2% Tween–PBS and added to the blots. The blots were allowed to react for 1 h at room temperature and then washed. The blots were finally developed in the H_2O_2 substrate and 4-chloro-1-naphthol.

N-terminal amino acid sequence determination. The 67-kDa protein was separated either from the λT.2.18 phage lysate or from the cleavage products of the maltose-binding protein (MBP)–66-kDa fusion protein by SDS–10% PAGE and electroblotted onto a polyvinylidene difluoride membrane. A part of the membrane was used for Western blot analysis with ARF sera to locate the protein. The rest of the membrane was stained with Coomassie blue, and the corresponding protein bands were cut out of the membrane and subjected to N-terminal amino acid sequence analysis with a 470A gas-phase protein sequencer equipped with a 120A on-line phenylthiohydantoin amino acid analyzer (Applied Biosystem, Inc.) in accordance with standard procedures (16).

DNA sequencing. Overlapping fragments of streptococcal DNA were cloned into M13mp18 or mp19 (39). Nucleotide sequences were determined by the dideoxy-chain termination method of Sanger et al. (32), with *Taq* DNA polymerase (Promega Co., Madison, Wis.) and universal primer M13. Synthetic oligonucleotide primers (Molecular Biology Resource Facility, Oklahoma University Health Sciences Center) were also used when streptococcal DNA fragments inserted into M13 were too large to sequence with the universal primer.

Sequence analysis. DNA sequence data were analyzed by the SEQ program (Intelligenetics). The amino acid sequences deduced from open reading frames (ORFs) were analyzed by the FASTA or FASTDB program (Intelligenetics) for homology with sequences in the data base and by PEP (Intelligenetics) to determine hydropathicity (20).

Purification of the recombinant 67-kDa protein. The recombinant 67-kDa protein was purified with the Protein Fusion & Purification System (New England BioLabs, Beverly, Mass.). The 67-kDa gene was fused in frame to the C-terminal region of the *malE* gene in the phagemid pMAL-2c vector. An about 2.3-kb *Hin*dIII fragment containing most of the coding sequence of the 67-kDa protein, except for the N-terminal eight amino acid residues, was inserted into the *Hin*dIII site of the pMAL-2c vector. The resulting recombinant plasmid, pKS53 (pMAL-c2), contained the *Hin*dIII fragment in the right orientation relative to the *malE* gene. The 67-kDa gene in the *Hin*dIII fragment was readjusted to the *malE* gene in the correct reading frame by deleting nucleotides in the multiple cloning sites. Plasmid pKS53 was digested with *Eco*RI and *Sal*I and then continuously with nuclease S1 to delete single-stranded DNA from the ends. DNA was purified by phenol-chloroform extraction followed by ethanol precipitation and then ligated overnight at 15°C. *E. coli* JM109 transformants were induced to express the fusion protein by adding IPTG to exponentially growing cells at a final concentration of 0.3 mM. Cells were grown for 4 to 5 more h after addition of IPTG and then collected by centrifugation. Expression of the fusion protein was examined by Western immunoblotting with rabbit anti-MBP serum. A transformant producing the fusion protein with the expected molecular weight was used to purify the 67-kDa protein. *E. coli* cells harvested from a 1-liter culture volume were resuspended in 50 ml of column buffer (10 mM Tris-Cl, 200 mM NaCl, 1 mM EDTA) and then sonicated for 15 min (40% duty, three 5-min pulses). The fusion protein was purified from crude *E. coli* cell extract on an amylose affinity column, concentrated at 1 mg/ml with Centriprep-10 (Amicon, Inc., Beverly, Mass.), and then cleaved into MBP and the 67-kDa protein by factor Xa, which specifically recognizes an amino acid sequence, I-E-G-R, right upstream from the 67-kDa protein. The 67-kDa protein was again purified from the cleavage products by using a DEAE-cellulose ion-exchange column. The N-terminal sequence of the purified 67-kDa protein was confirmed by automated protein sequencing as described above.

Enzyme-linked immunosorbent assay (ELISA). The recombinant 67-kDa protein was diluted in carbonate-bicarbonate buffer (pH 9.6) to a final concentration of 100 μg/ml, dispensed onto Immulon 4 microtiter plates, and incubated overnight at 4°C. After being washed three times with PBS-Tween 20 (0.05%), uncoated sites were blocked with 1% bovine serum albumin in PBS-Tween 20 by incubation for 1 h at 37°C. The plates were washed again three times with PBS-Tween 20. Anti-IA sera and normal mouse serum were diluted (1/50) in blocking buffer and added to each well in a volume of 50 μl. After overnight incubation, the plates were again washed three times with PBS-Tween 20 and anti-mouse polyvalent immunoglobulins conjugated with alkaline phosphatase (1:500 dilution in PBS) were added. The plates were incubated for 2 h at room temperature and then washed again with PBS-Tween 20; this was followed by development with a *p*-nitrophenyl phosphate substrate (1 mg/ml in diethanolamine buffer). The optical density at 405 nm was measured with a Dynatek MR700 ELISA reader.

Southern hybridization. A 1.2-kb *Hin*dIII-*Kpn*I fragment obtained from the internal sequence of the 67-kDa gene was radiolabelled with [α-^{32}P]dATP by nick translation. Streptococcal genomic DNA was digested with restriction enzyme *Kpn*I overnight at 37°C and fractionated on a 0.7% agarose gel. DNA was denatured for 30 min (2 × 15 min) in 1.5 M NaCl–0.5 M NaOH, neutralized in 1.5 M NaCl–1 M Tris-HCl (pH 7.4), and then transferred overnight to an S & S Nytran membrane (pore size, 0.2 μm; Schleicher & Schuell, Inc., Keene, N.H.) by the Southern technique (40). Hybridization and washing steps were performed at 68°C as described by the supplier.

RESULTS

Cloning of the gene encoding a streptococcal antigen reactive with ARF serum. A total of 11 lambda phage clones showed a positive signal for antigen production in phage lysate extracts with ARF serum in Western blots. Several different clones were identified on the basis of the molecular weight of the protein reacting with the antibody probe (data not shown). Recombinant phage T.2.18, containing the gene specifying the 67-kDa antigen, was selected for further study because of its strong reaction with the ARF antibody probe and the apparent molecular mass of approximately 60 kDa (Fig. 1). A 60-kDa antigen was originally sought, since a myosin-cross-reactive 60-kDa antigen had previously been identified in streptococcal membranes (3). The molecular size of the recombinant antigen expressed from λT.2.18 in *E. coli* was initially thought to be 60 kDa on the basis of SDS-PAGE protein standards until the gene was subcloned and sequenced and found to encode a

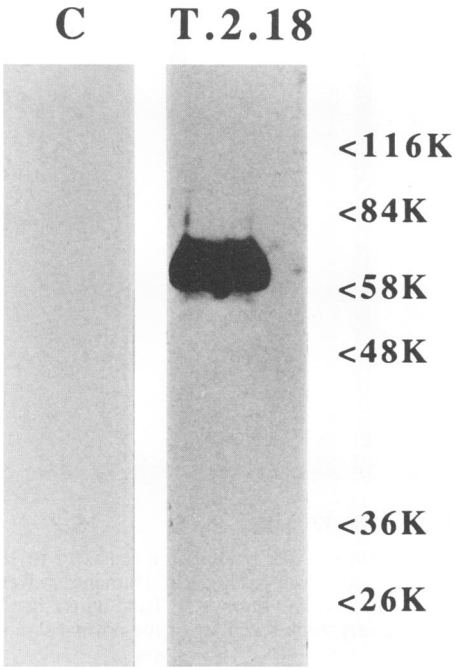

FIG. 1. Western blot of proteins from phage lysate of recombinant clone T.2.18 or host strain *E. coli* LE392 proteins (lane C) reacted with a selected ARF patient serum. Molecular size markers ranged from 116 to 26 kDa, as indicated on the right.

67-kDa protein. It is not known if the 60-kDa membrane antigen(s) previously described (3) is the same as the 67-kDa antigen described in this report. Antigens in *E. coli* and phage negative controls were not reactive with the ARF serum antibody probe.

All of the ARF sera tested revealed a strong positive reaction with the 67-kDa antigen (Table 1). Normal sera showed very weak or no reactivity against this antigen (Fig. 2). However, 10 individual sera from patients with uncomplicated streptococcal disease demonstrated strong positive reactivity with the 67-kDa antigen in the T.2.18 phage lysate. The results are shown collectively in Table 1. Furthermore, 15 of 19 sera from poststreptococcal AGN patients (with elevated ASO titers) were strongly reactive. Only sera demonstrating strong reactivity against the 67-kDa antigen, as shown with the ARF sera tested (Fig. 2), were reported as positive in Table 1. Myosin-specific antibodies purified from the original ARF serum reacted with a 67-kDa antigen in T.2.18 lysates but did not react with phage lysates from other clones (T.1 and T.24) (Fig. 3). Clones T.1 and T.24 were isolated in the initial screening of the streptococcal genomic library with an ARF

TABLE 1. Reactivity of sera from patients with streptococcal diseases and sequelae with the 60-kDa antigen

Disease	ASO titer range (Todd units)	No. of sera positive/ total no. tested by Western immunoblot
Uncomplicated streptococcal infection	250–333	10/10
ARF	320–>2500	20/20
AGN	120–>960	15/19
None	<50	1/20

serum and were found by Western blotting to produce proteins of 160 to 180 and 70 kDa, respectively. Although the reaction of the ARF serum with proteins expressed in T.1 and T.24 was weaker than the ARF serum reaction with T.2.18, Fig. 3 shows the very strong reaction of the ARF serum with the expressed 67-kDa protein in T.2.18 and the specificity of the myosin-specific antibodies for the 67-kDa protein. No reaction was observed with *E. coli* containing only phage DNA (data not shown). These results indicate that the recombinant protein encoded by clone T.2.18 has immunological epitopes in common with myosin, while other recombinant proteins recognized by the ARF serum do not. Plasmid pKS40, containing the 10-kb *Eco*RI-*Sal*I fragment of streptococcal DNA, produced a 67-kDa protein which was recognized by ARF sera (Fig. 4). Sequencing of about 4 kb of DNA located between the first *Xba*I site near the *Sal*I site and the *Pst*I site in pKS40 revealed two ORFs (Fig. 5). Eight amino acids starting from the fourth codon of the second ORF were identical to those (T-S-G-N-Y-E-A-F) determined by N-terminal amino acid sequencing of the 67-kDa protein purified from the λT.2.18 phage lysate. Although there was an inconsistency regarding the molecular size of the protein, these data showed that the 67-kDa protein (originally thought to be 60 kDa) expressed by λT.2.18 contained an N-terminal amino acid sequence identical to that deduced from the nucleotide sequence of the second ORF in pKS40. Hence, the molecular size of the streptococcal protein recognized by ARF sera was considered to be 67 kDa.

Nucleotide sequence of the gene encoding the 67-kDa streptococcal protein. The complete nucleotide sequence of the gene encoding the 67-kDa streptococcal antigen and its flanking regions and the deduced amino acid sequence of the gene are shown in Fig. 6. The ORF encoding the 67-kDa antigen spans the region between nucleotides 179 and 1952 and encodes a protein of 590 amino acids with a calculated molecular weight of 67,381. Although there is another ATG codon at nucleotides 140 to 142, the ATG codon at nucleotides 179 to 181 was chosen as the start codon for the 67-kDa protein because of its ideal location relative to the putative ribosome-binding site located 8 bp upstream of the start codon (25). The putative ribosome-binding site (AAGGAGG) is complementary to the 3′ end of *E. coli* 16S RNA. In addition, the nucleotide sequence downstream of this start codon seems to encode a signal peptide similar to other bacterial signal peptides, although it was not cleaved when expressed in *E. coli*. Upstream of the ORF, there are putative −35 and −10 promoter sequences, ATGATA and TATTAT, that resemble the consensus sequences previously described for a gram-positive promoter with an ideal spacing of 16 bp (5, 26). This sequence may function as a promoter; however, we do not know how the two ORFs reported here are controlled. It is possible that they are not part of a single operon, which would eliminate the need for a promoter directly upstream of the second ORF. Downstream of the TAA stop codon is a region of dyad symmetry that is capable of forming a stable stem-and-loop structure. The potential hairpin, which consists of a 12-bp stem separated by a 6-bp loop (ΔG, −16 kcal [1 cal = 4.184 J]), is followed by a stretch of T residues and may function as a ρ-independent terminator (30).

Deduced amino acid sequence of the 67-kDa streptococcal protein. On the basis of amino-terminal sequencing data and the putative start codon, no cleavable signal peptide was identified for the 67-kDa antigen, at least when it was expressed in *E. coli*. However, hydropathy analysis of the deduced amino acid sequence of the 67-kDa antigen revealed some characteristics of signal peptides. The 67-kDa antigen

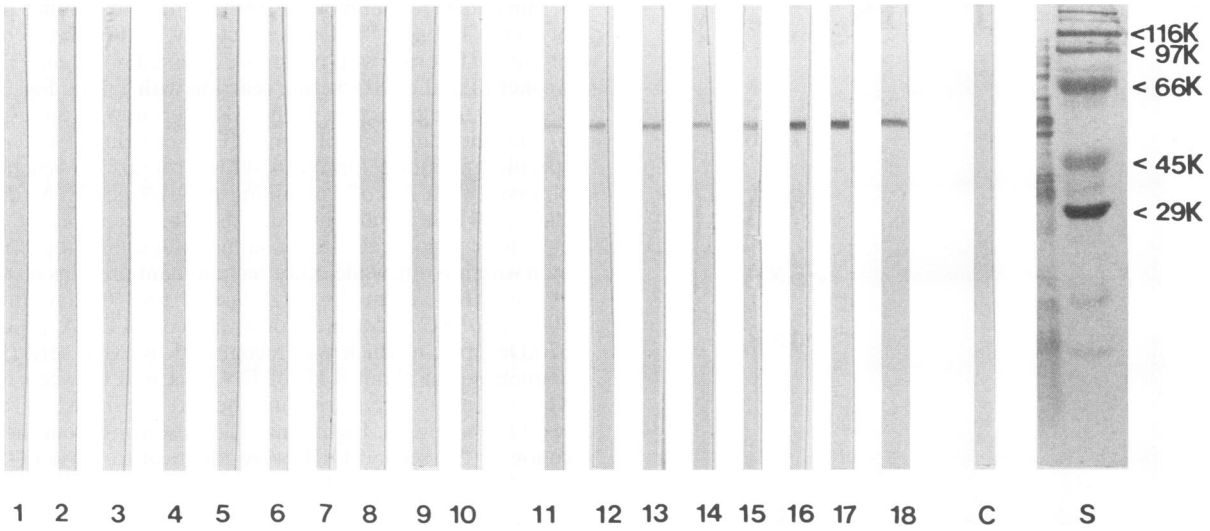

FIG. 2. Western blot of recombinant phage lysate reacted with normal sera (lanes 1 to 10) or ARF patient sera (lanes 11 to 18). Lane C, antibody conjugate control. The molecular size standards in lane S ranged from 116 to 29 kDa, as shown on the right. Human sera were collected at the time of disease manifestations from patients with confirmed ARF or from healthy controls (ASO titers, <50 Todd units). See Table 1 for an analysis of additional sera. Only sera demonstrating strong reactivity over and above the very weak reactivity of the normal sera shown here were considered positive.

starts with a hydrophilic amino terminus (positions 1 to 24) followed by a strong hydrophobic region (positions 25 to 42). In addition, the hydrophobic region is preceded by two positively charged residues (R and K; underlined in Fig. 6), which also support the presence of a signal peptide (37). Comparison of the deduced amino acid sequence of the 67-kDa antigen with those in data bases revealed similarity to the β chain of mouse MHC class II antigens (for example, haplotype u) (Fig. 7A). The similarity occurs over 151 amino acid residues with about 19% identity. If conservative amino acid substitutions are taken into consideration, the similarity is about 62%. The 67-kDa antigen also exhibits similarity to β chains of human class II antigens. Figure 7B shows the similarity between the human class II antigen DQ and the 67-kDa streptococcal antigen. Interestingly, two cysteine residues were found at residues 89 and 124, in the region of the 67-kDa antigen, demonstrating similarity to the MHC class II antigen(s). One of the cysteines (residue 124) was positioned identically to a cysteine (residue 104) in the β chain of the mouse MHC class II antigen. The hydropathic profiles of the homologous regions also exhibited a general similarity (Fig. 8). Although some alpha-helical regions may exist in the 67-kDa protein, it does not have the predicted alpha-helical coiled-coil structure found in myosins and M proteins.

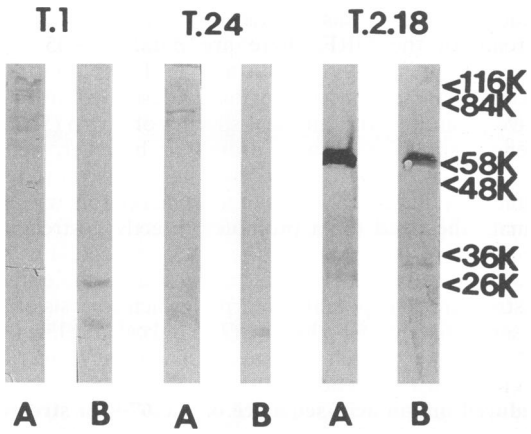

FIG. 3. Western blot of clone T.1, T.24, and T.2.18 phage lysates reacted with a single ARF serum (lanes A) or myosin affinity-purified antibodies from the same serum (lanes B). Molecular size markers ranged from 116 to 26 kDa, as shown on the right. The serum used was the same as that shown in Fig. 1 and 2; however, multiple sera were tested against clone T.2.18, as shown in Fig. 2 and described in Table 1.

Presence of the gene for the 67-kDa antigen in different groups of streptococci. A Southern hybridization experiment (Fig. 9) revealed that a 1.2-kb radiolabelled *Hin*dIII-*Kpn*I fragment obtained from the internal sequence of the gene for the 67-kDa antigen hybridized with DNAs from all of the serotypes (M24, M5, and M−) of group A, C, and G streptococci tested. DNAs from *S. aureus*; streptococcal groups B, D, P, and R; and *S. mutans* did not hybridize to the probe. Except for the serotype M5 strain, all of the positive strains produced a single band (1.8 kb) identical to that of the M− strain from which the gene for the 67-kDa antigen was cloned.

Reaction of anti-IA sera with the purified 67-kDa protein. Seven anti-IA sera (prepared against a group of haplotypes) obtained from the National Institutes of Health repository were reacted with the purified 67-kDa protein. The 67-kDa protein was expressed as an MBP–67-kDa fusion protein and was cleaved free of MBP by factor Xa. The cleavage products were separated by DEAE-cellulose ion-exchange column chromatography, and the purified 67-kDa protein cleavage product is shown in Fig. 10. The 67-kDa cleavage product contained only that protein, since the major and minor bands demonstrated 67-kDa amino acid sequences upon analysis. The reaction of the anti-IA sera with the 67-kDa protein is shown in Fig. 11. The anti-IA serum reactions were about five times as strong as the reaction of normal control mouse serum. No reaction was observed when serum was reacted with MBP as a control protein (data not shown).

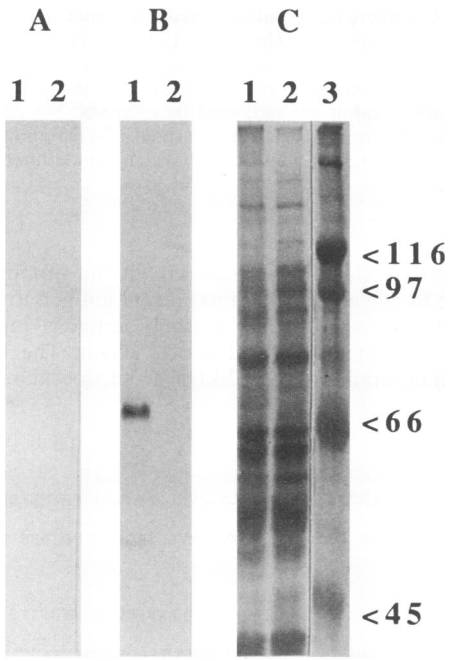

FIG. 4. Expression of the 67-kDa streptococcal protein from *E. coli* harboring plasmid pKS40. SDS lysates of *E. coli* were separated by SDS–8% PAGE and subjected to Western immunoblotting with ARF (B) or normal (A) serum. Four different ARF and normal sera were tested against the pKS40 proteins with similar results. The results shown were obtained with the original serum used for cloning, but all of the ARF sera were strongly positive. The normal serum shown showed slight reactivity, as shown in Fig. 2 for normal sera. Part of the blot was subjected to amido black protein staining (C). Lanes: 1, pKS40; 2, pUC19; 3, molecular size markers in kilodaltons.

DISCUSSION

ARF and rheumatic carditis are serious complications of *S. pyogenes* pharyngitis. Although the pathogenesis of ARF is unknown, there is strong evidence of immunologically mediated mechanisms (17, 18, 41, 42). An exaggerated antibody response to streptococci and heart tissue is characteristic of ARF, with antibody and complement deposition detected in the heart at autopsy (18, 42). A proposed mechanism of cardiac involvement in ARF is development of streptococcal antibodies that cross-react with heart tissue. Streptococcal antigens have been shown to react with anti-heart antibodies, including the streptococcal cell walls (17) and M proteins (9, 12, 13), as well as components in the streptococcal membrane (8, 36, 41). The components in the streptococcal membrane which elicit heart-reactive antibodies have not been identified, and it has been our goal to investigate and identify antigens other than the M protein which might be important in eliciting a heart-reactive antibody response in ARF. For this reason, we used an ARF serum to screen streptococcal DNA libraries in λEMBL3. Although we used a single serum to screen the library, we found that all of the ARF sera tested reacted strongly with the 67-kDa antigen. We originally thought that the 67-kDa antigen might be associated only with ARF, but we have found that all individuals with streptococcal infections and/or sequelae respond strongly to the 67-kDa antigen. Healthy individuals without streptococcal infection do not have high levels of antibody against the 67-kDa antigen, although some low level of antibody is most likely present in normal sera. These findings are not really surprising, since most individuals are expected to respond in general to strong bacterial antigens. Therefore, the presence of antibody against the 67-kDa antigen in sera from patients with streptococcal diseases suggests that it is associated with host responses against group A streptococcal infection. Further studies are in progress to determine the differences between responses in patient groups both qualitatively and quantitatively. The role of the 67-kDa antigen in the development of ARF or other autoimmune phenomena related to infection is not clear. However, it is clear that the 67-kDa antigen reacted with anti-myosin antibodies affinity purified from individual ARF sera. Although we did not screen large numbers of affinity-purified anti-myosin antibodies against the 67-kDa protein, our past studies suggest that idiotypic determinants on anti-myosin antibodies in ARF and AGN sera are similar, as are their specificities for myosin and M protein (9, 24). On the other hand, anti-myosin antibodies in normal sera are present at low levels and differ in specificity (9, 10). Further studies on the specificity of anti-myosin antibodies affinity purified from ARF sera and sera from patients with other streptococcal diseases is in progress.

Although the 67-kDa antigen was shown to react with anti-myosin antibodies, the deduced amino acid sequence of the 67-kDa antigen was not found to have a large amount of sequence identity to myosin or any known streptococcal protein. This may indicate that anti-myosin antibodies recognize conformational epitopes common on both proteins. On the other hand, the protein data base comparison did show a significant amount of identity and similarity to the class II MHC molecules of mice and humans. The similarity in the overlapping hydropathic profiles of the homologous regions of the two molecules was impressive, but there was no identity

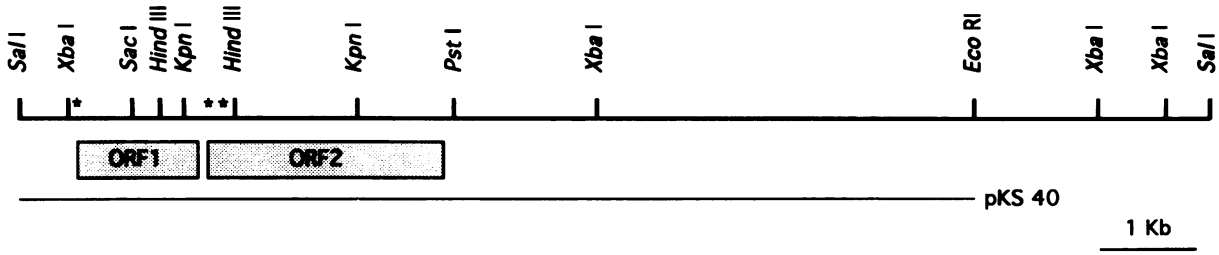

FIG. 5. Restriction map of the 12.5-kb streptococcal DNA in clone λT.2.18 and plasmid subclone pKS40. The solid line represents the streptococcal DNA insert. The original λT.2.18 clone contains the 12.5-kb *Sal*I fragment. Plasmid pKS40 was constructed by substituting the 10-kb *Sal*I-*Eco*RI fragment of streptococcal DNA for the corresponding segment in the multiple cloning site of pUC19. The asterisks indicate putative promoter sites for the corresponding downstream genes. ORF2 encodes the 67-kDa streptococcal protein.

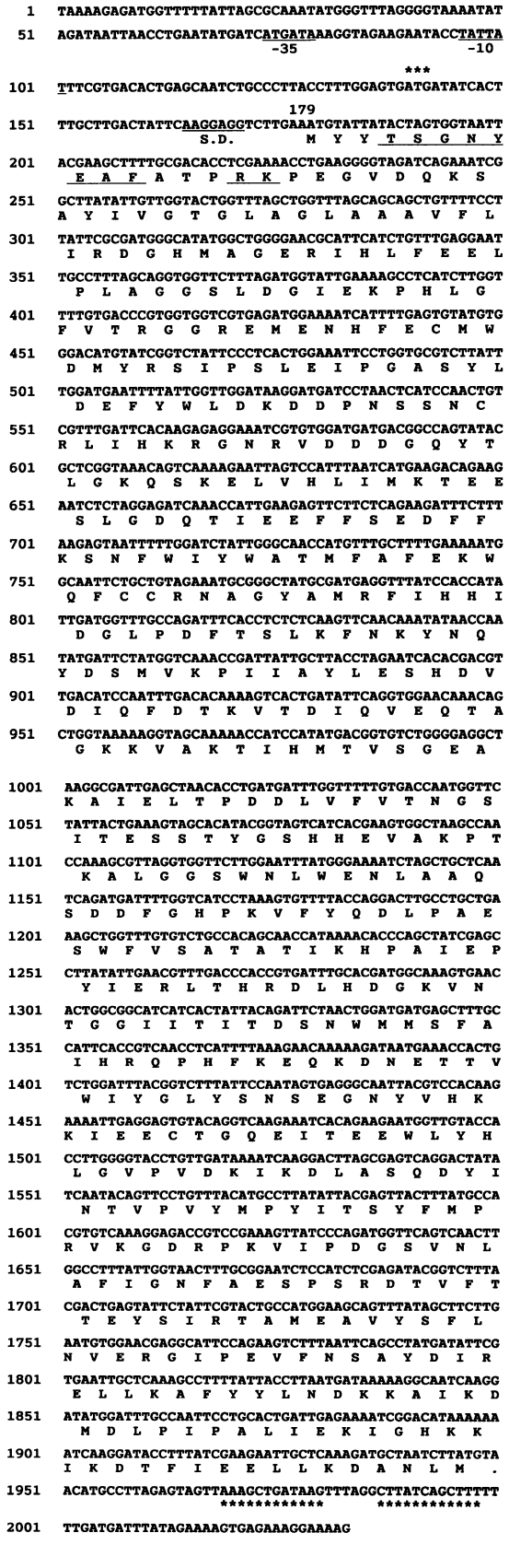

FIG. 6. Complete nucleotide and deduced amino acid sequences of the 67-kDa streptococcal antigen. The putative ribosome-binding site (S.D.) and the −35 and −10 regions of the putative promoter sites are underlined. The putative termination signal and the alternative translation initiation codon are indicated by asterisks. N-terminal amino acid residues determined from the purified 67-kDa antigen and two positively charged residues (R and K) are also underlined.

(Fig. 8). MHC class II antigens are in the immunoglobulin supergene family and function to present antigen to T lymphocytes (28). The structure of class II antigens involves two proteins, an alpha chain and a beta chain. The beta chain appears to be similar to the 67-kDa streptococcal protein. The

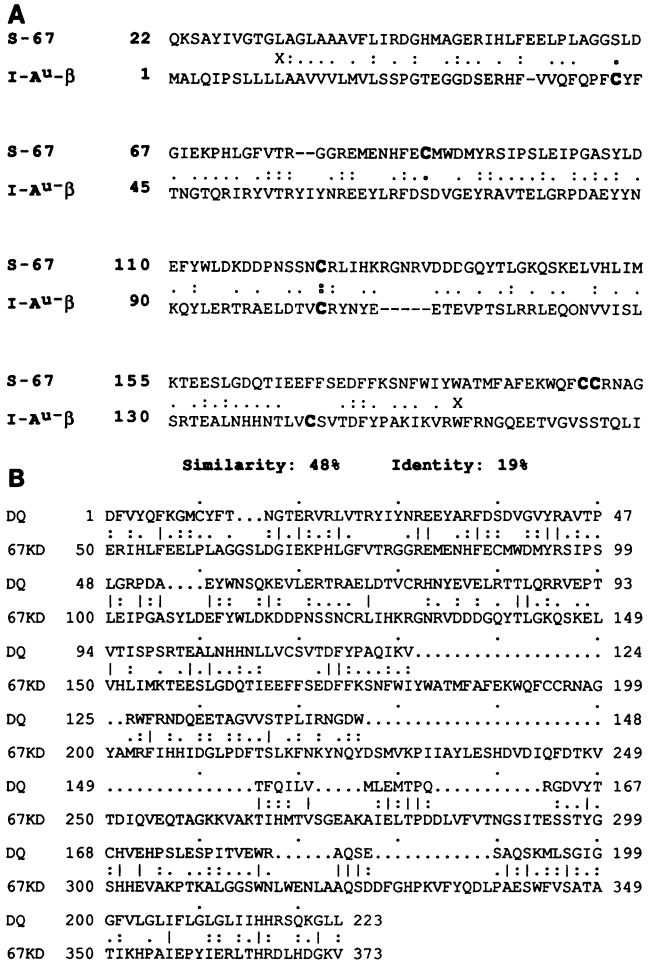

FIG. 7. (A) Amino acid sequence homology between the N-terminal region of the streptococcal 67-kDa antigen (S-67) and the β-1 domain of the mouse I-A^u antigen (I-A^u-β). The primary sequences were aligned by using the program FASTA (Intelligenetics). Double dots represent identity, and single dots represent conservative amino acid substitutions. Cysteine residues essential for the formation of disulfide bonds are outlined. (B) Amino acid sequence homology between the N-terminal region of the 67-kDa antigen and the β chain of the human DQ antigen. The primary sequences were aligned by using the Bestfit program of the Gencore protein data base, Genetics Computer Group computer programs. Lines represent identities, double dots represent conserved substitutions, and single dots represent functional substitutions.

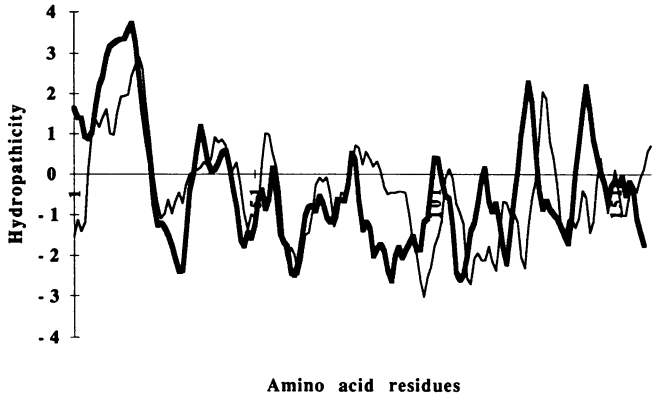

FIG. 8. Hydropathic profile comparison of the homologous regions of the 67-kDa antigen and the β-1 domain of the mouse I-A^u antigen. The dotted line represents the I-A^u β chain, and the bold line represents the 67-kDa antigen. Values on the *x* axis represent numbered amino acid residues of the β chain.

MHC class II beta chain is known to contain two disulfide loops. The presence of cysteine residues in similar sites within the two molecules was an interesting observation. The entire 67-kDa protein contains five cysteines, with two of them residing side by side in the molecule. Preliminary data suggest immunological cross-reactivity between the 67-kDa antigen and MHC class II molecules, since anti-IA sera obtained from the National Institute of Allergy and Infectious Diseases (Bethesda, Md.) reacted with the purified 67-kDa antigen. Further studies are in progress to determine if the 67-kDa antigen elicits antibodies against class II MHC molecules. Furthermore, it is not known if patients with ARF produce antibodies against class II MHC molecules or if anti-myosin antibodies may cross-react with class II antigens. It is possible

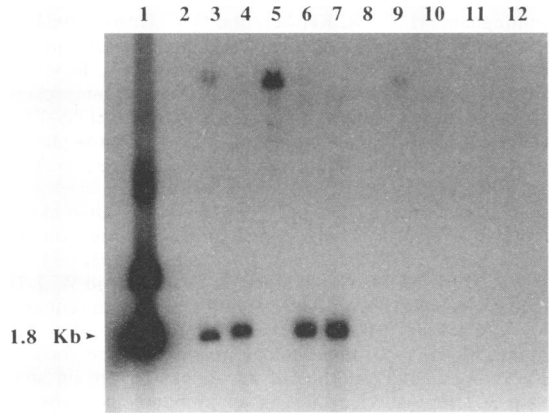

FIG. 9. Southern blot analysis of different strains of streptococci. Chromosomal DNAs isolated from different strains were digested with *Kpn*I, fractionated by electrophoresis on a 0.7% agarose gel, subjected to Southern blotting, and probed under high-stringency conditions with the 67-kDa gene probe. The probe DNA was prepared by nick translation of the 1.2-kb *Hin*dIII-*Kpn*I fragment obtained from the internal sequence of the 67-kDa gene. Lanes: 1, plasmid marker DNA for the 1.8-kb *Kpn*I fragment; 2, *S. agalactiae* (group B); 3, *S. pyogenes* M24 (group A); 4, *S. pyogenes* M− (group A); 5, *S. pyogenes* M5 (group A); 6, streptococcal group G; 7, *S. equisimilis* (group C); 8, streptococcal group R; 9, streptococcal group P; 10, *S. aureus*; 11, *S. faecalis* (group D); 12, *S. mutans*.

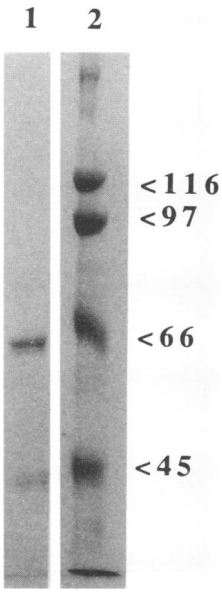

FIG. 10. Purification of the 67-kDa protein. The cleavage products of the fusion protein were separated by DEAE-cellulose ion-exchange chromatography. Twenty microliters from a peak of the purified 67-kDa protein-containing fractions was mixed with an equal volume of 2× 5% SDS–cracking buffer, boiled for 10 min, and then loaded onto an SDS–8% polyacrylamide gel. Proteins were detected by Coomassie blue staining. Lanes: 1, 67-kDa protein; 2, molecular weight standards (10^3). Both protein bands contained the amino acid sequence of the 67-kDa protein.

that antibodies against the 67-kDa antigen react with sites in inflamed tissues, where expression of class II MHC molecules is upregulated (2).

It is not known if the 67-kDa antigen plays a role in the pathogenesis of streptococcal infections. However, the gene encoding the 67-kDa antigen was found only in streptococcal groups A, C, and G, which are known pathogenic groups of streptococci (6, 35). Streptococcal groups C and G are also known to contain a gene similar to *emm* which encodes the M protein of group A streptococci (6, 15, 33). Since it has been suggested that the M protein is associated with other proteins (38), the question of whether the 67-kDa antigen is associated with the M protein is of interest. The location of the 67-kDa protein is not known, but the protein is believed to be present in the walls and membranes of group A streptococci. Western blot analysis of the membrane fraction prepared from a mutant with an insertionally inactivated 67-kDa gene suggested that the 67-kDa antigen is not identical to the 60-kDa wall-membrane antigen recently described by Barnett and Cunningham (3). However, it is still uncertain if anti-myosin antibodies in ARF sera react with more than one protein with a molecular mass of 60 kDa. Further studies are needed to determine if these two antigens are the same.

The nucleotide sequence data exhibit the structural gene of the 67-kDa antigen with all of the signals necessary for transcription and translation which are located within the 5-kb *Sac*I-*Xba*I fragment of streptococcal DNA. We also found an additional ORF upstream of the 67-kDa gene (Fig. 5).

The amino-terminal sequence of the 67-kDa antigen seems to have some characteristics in common with signal peptides. It begins with an N-terminal hydrophilic domain followed by a central hydrophobic domain and, in turn, by a polar C termi-

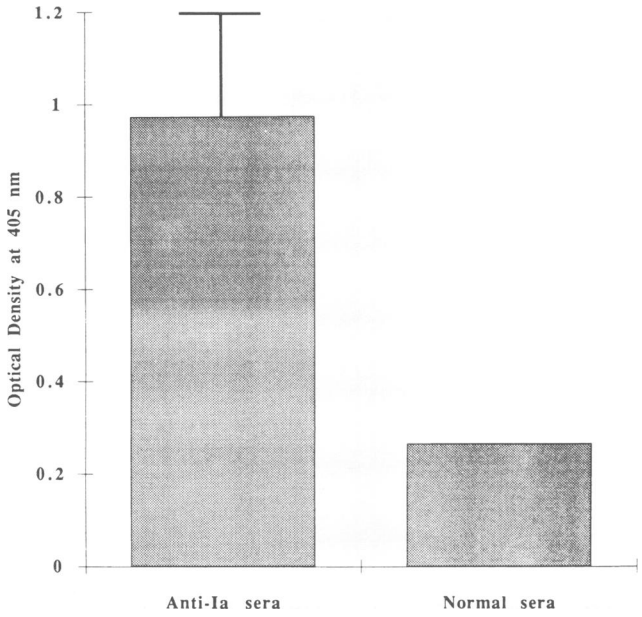

FIG. 11. Reactivity of the purified 67-kDa protein with antisera against mouse class II MHC antigens (Ia) in an ELISA. The purified 67-kDa protein reacted with six different mouse anti-IA sera five times more strongly than did normal mouse serum. Mouse anti-IA sera were obtained from the National Institute of Allergy and Infectious Diseases repository and were prepared against several different haplotypes of mouse IA antigen. All sera were diluted (1/50) in 1% bovine serum albumin–PBS–Tween buffer before reaction with the 67-kDa antigen in the ELISA. Normal mouse serum was a pool from matched strains of mice.

nus. Positively charged amino acids are also located in front of the hydrophobic domain. These positively charged amino acids have been suggested to play a role in the interaction between the preprotein and SecA (1). Nevertheless, the 67-kDa antigen revealed no cleavable signal peptide when expressed in *E. coli*. This may be due to lack of a consensus sequence at the C terminus, which is necessary for cleavage by a peptidase (29, 34). Alteration in the consensus sequence causes proteins which would otherwise be secreted to be anchored to the membrane (29). It has also been suggested that a hydrophobic domain located at the extreme N terminus is enough for export of the protein (29). These observations suggest that the 67-kDa antigen is a membrane protein with a signal peptide which is not cleaved by a peptidase after translocation of the protein.

In summary, we present the cloning and sequence analysis of a streptococcal gene which is present only in pathogenic streptococci and encodes a protein which reacts with anti-myosin antibodies from ARF and is similar to class II MHC molecules.

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