Expression of the Arp Protein, a Member of the M Protein Family, Is Not Sufficient To Inhibit Phagocytosis of *Streptococcus pyogenes*

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Many *Streptococcus pyogenes* immunoglobulin-binding proteins have structural similarities to the antiphagocytic M protein, including the well-known C repeats. One of these molecules is the immunoglobulin A-binding protein Arp, which is expressed by a serotype 4 strain for which no antiphagocytic M protein has yet been described. We expressed Arp4 in an *S. pyogenes* strain from which the structural gene for the M protein has been deleted and found that Arp4 is not sufficient to inhibit phagocytosis.

The group A streptococcus (*Streptococcus pyogenes*) is a common human pathogen capable of producing a variety of acute infections, including pharyngitis and impetigo. In addition, rheumatic heart disease or acute glomerulonephritis sometimes develops after a streptococcal infection. In recent years, a severe toxic shock-like syndrome caused by *S. pyogenes* has also been described (2, 33, 38).

The M protein, which is an important virulence factor of *S. pyogenes*, protects the organisms from phagocytosis by polymorphonuclear leukocytes (19). The mechanism for this protection is not known in detail, but it may involve interference with complement deposition on the bacterial surface. More specifically, it has been suggested that M protein inhibits activation of complement via the alternative pathway (5, 27), possibly through binding of complement factor H (17) or fibrinogen (34, 35) to the M⁺ bacterial cell surface.

In addition to M proteins, immunoglobulin (Ig)-binding proteins with affinity for IgG and/or IgA are commonly found on the surface of *S. pyogenes* strains (3, 18, 22). These molecules also have been implicated in virulence (7). Many of the Ig-binding proteins of *S. pyogenes* have structural similarities to M proteins and are therefore classified as members of the M protein family (13, 14, 16, 24). However, an M protein is defined by its ability to protect the organisms from phagocytosis (19), a property that has not yet been demonstrated for any of the proteins initially characterized as Ig-binding proteins, which have therefore been referred to as M-like or M-related proteins (4, 24). Two of the classical M proteins, the M1 and M12 molecules, have recently been reported to bind IgG (1, 30), indicating that some M and M-like proteins have both an antiphagocytic and an Ig-binding function.

The Ig-binding proteins of the *S. pyogenes* serotype 4 strain AP4 have been studied in some detail. One of these proteins, called Mrp4 (for M-related protein), is an IgG-binding protein which also binds fibrinogen (20, 32), as do the classical M proteins (36, 37). The Mrp4 molecule is a member of the M protein family but shows only limited sequence homology to the classical M proteins and lacks the well-known C repeats (24). The gene encoding Mrp4 is located immediately upstream of a gene encoding a second Ig-binding protein desig-

nated Arp4 (for IgA receptor protein). Arp4 binds IgA of both subclasses with high affinity and also binds polyclonal IgG weakly, but it does not bind fibrinogen (21, 32). Unlike the Mrp4 protein, the Arp4 molecule shows extensive sequence homology to M proteins, in particular in the C-terminal half of the molecule, and there are three C repeats in the central part of the molecule. In addition, Arp4 exhibits a 7-residue periodicity in the placement of nonpolar amino acids which is characteristic of alpha-helical coiled-coil proteins like M proteins (23). However, this periodicity is not as extensive in Arp4 as it is in Mrp4 or M proteins.

The goal of this study was to determine whether the Arp4 protein has an antiphagocytic function, since it has C repeats and shows extensive sequence homology to M proteins. Very little is currently known about the biological properties of the different Ig-binding proteins, but it seems likely that the identification of their functions will contribute to our understanding of the mechanisms that allow strains of *S. pyogenes* to cause disease.

Bacterial strains and plasmid constructions. Plasmid pARP401 (13) contains the entire *arp4* gene of *S. pyogenes* AP4 (21) cloned into pUC18. Plasmid pJRS264 was constructed by inserting the blunted 2.2-kb *Bam*HI-*Hind*III fragment of pARP401 into the blunted *Bam*HI site of plasmid pLZ12Spec, a vector capable of replication in both *Escherichia coli* and *S. pyogenes*. pLZ12Spec was constructed from pLZ12 (8) by ligating the blunted 1.7-kb *Pvu*II-*Nde*I fragment of plasmid pDL269 (9), containing its spectinomycin resistance gene, to the blunted 2.2-kb *Stu*I-*Sal*I fragment of pLZ12. *S. pyogenes* JRS145, derived from the M6 strain D471 (31), has *cat86* substituted for the M6 structural gene (*emm6.1*) (6). *S. pyogenes* JRS145(pJRS264) was constructed by electroporation (25).

PCR analysis. PCR was used to verify that the entire *arp4* coding region is present in strain JRS145(pJRS264). For use in PCR, total DNA was isolated from *S. pyogenes* AP4, JRS145 (pLZ12Spec), and JRS145(pJRS264) as previously described (10). The location of each of the PCR primers used is shown in Fig. 1A, and their sequences are as follows: 1A (24-mer), 5'-G CGGAGATTAAAAAGCCTCAGGCT-3'; 2 (21-mer), 5'-CA TTATCAAGAACAACAAAAG-3'; 3 (21-mer), 5'-AATTGT TGTGTCTTCTTGCTGA-3'; 4 (20-mer), 5'-CCAAACACCAA ACGCTAAAG-3'; 5 (20-mer), 5'-GCTGATCTAGAACGGT TAGC-3'; and "all M" reverse (33-mer), 5'-GGGGGGGAAT TCAGCTTAGTTTCTTCTTTGCG-3' (28). Amplifications

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FIG. 1. PCR analysis of strain JRS145(pJRS264). (A) The location of each of the synthetic primers used in this study is delineated by an arrow. rev., reverse. (B) PCR amplification. Lane M, molecular weight marker (100-bp DNA ladder; Gibco BRL, Grand Island, N.Y.); lane A, JRS145(pLZ12Spec) template, primers 1A and 3; lane B, AP4 template, primers 1A and 3; lane C, JRS145(pJRS264) template, primers 1A and 3; lane D, JRS145(pLZ12Spec) template, primers 2 and 5; lane E, AP4 template, primers 2 and 5; lane F, JRS145(pJRS264) template, primers 2 and 5; lane G, JRS145(pLZ12Spec) template, primers 4 and "all M" reverse; lane H, AP4 template, primers 4 and "all M" reverse; lane I, JRS145(pJRS264) template, primers 4 and "all M" reverse.

with primers 1A and 3 were performed in 50 μ l of a cocktail consisting of 3.5 mM $MgCl_2$, 60 mM Tris-HCl (pH 9.5), 15 mM $(NH_4)_2SO_4$, 200 μ M each deoxynucleoside triphosphate (dNTP), 0.4 μ M each primer, and 1.25 U of *Taq* polymerase (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). Twenty-five nanograms (total DNA) of template was added to each reaction mixture. Amplifications with primer pairs 2/5 and 4/"all M" reverse were performed in 50 μ l of a cocktail consisting of 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.3), 0.05% Tween 20, 0.05% Nonidet P-40, 200 μ M each dNTP, 0.8 μ M each primer, and 1.25 U of *Taq* polymerase. Fifty nanograms (total DNA) of template was added to each reaction mixture. Amplifications were carried out in a PTC-100 Programmable Thermal Controller (MJ Research, Inc., Watertown, Mass.), using 30 cycles consisting of 1 min of denaturation at 94°C, 1 min of annealing at 51°C, and 3 min of extension at 72°C. However, for the reactions with primers 1A and 3, the annealing temperature was increased to 55°C. The PCR products were visualized by electrophoresis of a 20- μ l volume for 5 h at 40 V in a 4% agarose gel containing 0.5 μ g of ethidium bromide per ml. The results are shown in Fig. 1B. For all reactions, the size of the amplified DNA fragment produced from strain JRS145(pJRS264) appears to be identical to that of the fragment produced from the wild-type strain AP4. In addition, all of the products are of the size expected on the basis of the published sequence of the *arp4* gene (13). These results indicate that the entire *arp4* coding region, from the N terminus of the mature protein through the putative membrane-anchoring domain, is present on the plasmid carried by strain JRS145(pJRS264).

Western immunoblot analysis. To confirm that the *arp4* gene is expressed by strain JRS145(pJRS264), Western immunoblot analysis was performed on lysin extracts (12) with rabbit anti-Arp4 antiserum (20). Two bands with approximate molecular masses of 41 and 44 kDa were detected in the lysate

obtained from JRS145(pJRS264) but not in the lysate obtained from the negative control (JRS145 carrying the vector alone) (data not shown). These sizes correspond well to those observed previously when the Arp4 protein was expressed in *E. coli* (13). In addition, multiple bands of higher molecular weight were present. These bands probably represent Arp4 protein bound to streptococcal cell wall fragments (12).

Ig and fibrinogen binding. The Western immunoblot analysis indicated that the Arp4 protein is produced by strain JRS145(pJRS264). To ascertain the role, if any, of Arp4 in conferring resistance to phagocytosis, it was also necessary to show that the Arp4 protein is localized to the surface of the organisms and is in a functional form. The binding of 125 I-labeled human fibrinogen and human polyclonal IgG and IgA to JRS145(pJRS264) was therefore analyzed, as previously described (21). At all bacterial concentrations tested, the degree of IgA binding by strain JRS145(pJRS264) was equivalent to that of the wild-type strain AP4 (Fig. 2). Furthermore, JRS145(pJRS264) bound polyclonal IgG weakly and did not bind fibrinogen (Fig. 2). This is the pattern of binding expected for an organism expressing only the Arp4 protein (21, 32). As expected, the negative control strain, JRS145(pLZ12Spec), did not bind any of the radiolabeled proteins. Evidence for surface expression of Arp4 was also obtained in experiments with anti-Arp4 serum, which reacted with strain JRS145(pJRS264) but not with the control strain (data not shown). These results clearly show that functional Arp4 protein is expressed on the surface of strain JRS145(pJRS264).

Phagocytosis assay. The ability of strain JRS145(pJRS264) to resist phagocytosis was assessed as previously described (26). Briefly, bacteria in the logarithmic phase of growth were mixed with THY (26) broth and freshly drawn whole human blood or plasma from the same donor and were incubated with rocking for 3 h at 37°C. The number of colony-forming units was determined by plating on THY agar. As expected, the M4 wild-type strain AP4 grew well in both whole blood and plasma compared with the blood-free medium control (Table 1). This indicates that this strain is resistant to phagocytosis and that serotype M4-specific antibodies were not present in the plas-

FIG. 2. Binding of ^{125}I -labeled human polyclonal IgA, human polyclonal IgG, and human fibrinogen to the wild-type strain AP4, the M-protein-negative control strain JRS145(pLZ12Spec), and the experimental strain JRS145(pJRS264), a transformant of JRS145 harboring the gene for the Arp4 protein. The binding of ^{125}I -labeled proteins was measured as a function of bacterial concentration, and each datum point is the average of duplicate samples. The data presented are representative of three independent experiments.

ma from these donors. The negative control strain, JRS145 (pLZ12Spec), grew in the presence of plasma but was phagocytosed in whole blood. Strain JRS145(pJRS264), which expresses Arp4, grew well in plasma but was phagocytosed in whole blood. Therefore, the Arp4 protein is not able to confer resistance to phagocytosis when expressed on the surface of an *S. pyogenes* strain which lacks M protein. This finding focuses attention on the role of Mrp4 in protection from phagocytosis.

The possibility remains that the Arp protein could play some role in virulence other than conferring resistance to phagocytosis. Bessen and Fischetti (3) found that nearly all *S. pyogenes* isolates from cases of impetigo bound human IgG, raising the possibility that IgG binding may be required for streptococcal pyoderma. A subsequent study by Raeder and Boyle (29) suggested that, in mice, organisms recovered from the spleen following lethal skin infection express significantly more IgG-binding proteins than do organisms recovered from the spleen following intraperitoneal inoculation with the same strain. Therefore, it appears that Ig-binding protein expression may contribute to invasiveness or may confer a unique selective advantage for survival at a particular site. A role for the Ig-binding proteins in virulence is also supported by the common occurrence of these proteins among clinical isolates of *S. pyogenes*, by the structural similarity to M proteins, and by the presence of amino-terminal sequence variation, suggesting that these proteins show antigenic variation due to a selective

pressure (11, 15). However, further studies are needed to demonstrate a precise biological function for the Ig-binding proteins in different types of group A streptococcal infections.

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TABLE 1. Survival in whole blood

Blood donor	Strain	CFU/ml			
		Input	Whole blood	Plasma	Medium control
1	AP4	6.5×10^2	8.4×10^3	3.3×10^4	5.0×10^3
	JRS145(pLZ12Spec)	3.5×10^2	$<1 \times 10^1$	2.2×10^3	2.6×10^3
	JRS145(pJRS264)	4.6×10^2	$<1 \times 10^1$	4.3×10^3	5.1×10^3
2	AP4	5.0×10^2	2.3×10^4	5.6×10^4	6.2×10^3
	JRS145(pLZ12Spec)	3.7×10^2	$<1 \times 10^1$	4.1×10^3	8.6×10^3
	JRS145(pJRS264)	4.9×10^2	$<1 \times 10^1$	3.0×10^3	7.7×10^3

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