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Evidence for Actinlike Proteins in an M Protein-Negative Strain of *Streptococcus pyogenes*

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Antigens shared between *Streptococcus pyogenes* and heart tissue may play an important role in autoimmune cardiac injury associated with acute rheumatic fever. Antiheart/antistreptococcal antibodies found in the disease react with antigens of *S. pyogenes*, including M protein and a 60-kDa antigen distinct from M protein. Heart antigens recognized by these cross-reactive antistreptococcal antibodies include myosin and actin. To investigate the presence of a streptococcal actin, established protocols for the polymerization and isolation of eukaryotic actin were used to extract and concentrate actinlike proteins from M$^-$ streptococcal cells. The polymerized bacterial actin from the streptococcal extract was probed in immunoblots with an antiactin monoclonal antibody. Two proteins of about 60 kDa in the polymerized bacterial actin reacted with the antiactin antibody. Proteins in the polymerized bacterial actin extract of about 43 and 60 kDa behaved like eukaryotic actin by binding to myosin and DNase I affinity columns. Filaments were demonstrated by electron microscopy in the polymerized bacterial actinlike extract, which also enhanced the ATPase activity of eukaryotic myosin. The data suggest that proteins resembling actin are present in *S. pyogenes*.

Streptococcus pyogenes pharyngitis is associated with the development of acute rheumatic fever, in which an exaggerated immune response to the streptococci is believed to play an important role in the pathogenesis of the disease (2, 32, 35). Further evidence to support the autoimmune development of acute rheumatic fever includes the presence of autoantibodies in serum samples from patients with acute rheumatic fever which react with streptococcal and heart proteins. The characterized host and streptococcal proteins targeted by the cross-reactive immune response are generally found to be highly conserved and functionally important proteins (4–8, 11–13).

Much of our previous work has dissected the cross-reactive immune responses to streptococcal infection which could lead to heart injury (5–10, 12). To study the immunological cross-reactivity between streptococci and heart tissue, human and mouse monoclonal antibodies (MAbs) were produced, characterized (5–10, 12, 16), and found to react with streptococcal and heart proteins. Two cross-reactive MAbs, 36.2.2 and 54.2.8, have been found to react in the host with numerous cytoskeletal proteins, including actin, myosin, tropomyosin, vimentin, and keratin, and in the streptococci with M protein and a 60-kDa protein distinct from M protein (1, 5–10, 12, 16). A common feature of these shared proteins is their highly conserved nature and their important function in the organism. The streptococcal M protein has been shown to possess a coiled-coil structure resembling those of myosin and tropomyosin and other coiled-coil structures (13, 19–21).

Contractile proteins such as actin are believed to exist in prokaryotes. Actinlike proteins have been reported for *Mycoplasma* spp. (24), *Escherichia coli* (22, 23), and halophilic archaebacteria (30). Functions for prokaryotic actins have included motility in the absence of locomotor appendages and maintenance of cell shape without a cell wall in studies of *Mycoplasma* spp. (24). In *E. coli*, actin has been proposed to account for cell swelling and contraction cycles accompanying potassium uptake (22, 23). Actinlike proteins have not been reported previously for *S. pyogenes*, although streptococcal M protein has been found to cross-link eukaryotic actin and inhibit myosin ATPase activity (3). The α-helical coiled-coil M protein has been characterized extensively (13, 15, 19–21), but a function for it in the streptococci other than the important role it plays in avoiding the immune system of the host has not been determined.

The immunological relationship already shown between streptococcal proteins and actin has prompted further investigation of the existence of streptococcal proteins with immunological and functional similarities to actin. The goal of this study was to define actinlike proteins in *S. pyogenes*. The data described support previous findings that prokaryotic organisms have actinlike elements, and this is the first report defining actinlike proteins in streptococci.

To alleviate the interference of M protein cross-linking of actin in our study, an M protein-negative (M$^-$) strain of *S. pyogenes* was investigated. The M protein-negative strain has a portion of its chromosomal DNA deleted, including the M protein gene, as described previously (28, 29). Whole cells of *S. pyogenes* M$^-$ were extracted by protocols for isolating and concentrating eukaryotic actin (25). For comparison, eukaryotic actomyosin from rabbit skeletal muscle was extracted simultaneously. M$^-$ streptococci were grown in chemically defined medium (34) to the exponential phase, and the cells were collected by centrifugation (10,000 × *g*, 10 min). The streptococcal cell pellet was sequentially extracted with stirring at 4°C with 0.1 M KCl for 7 min, 1 mM EDTA (pH 7.0) for 7 min, and acetone for 3 h. The extracted streptococcal cells were dried overnight onto Whatman no. 1 filter paper, and the resulting streptococcal acetone powder was stored desiccated at −20°C. Streptococcal acetone

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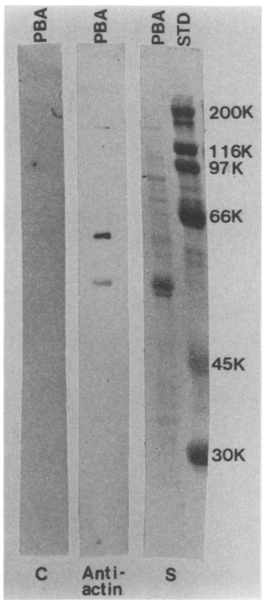

FIG. 1. Western blot of PBA from whole M⁻ *S. pyogenes* cells reacted with antiactin MAb (Dupont-NEN), a phosphate-buffered saline control (C), and a Coomassie blue protein stain (S). Protein size standards (lane STD; rabbit muscle myosin, β-galactosidase, rabbit muscle phosphorylase *b*, bovine serum albumin, egg albumin, and carbonic anhydrase) are shown (in kilodaltons).

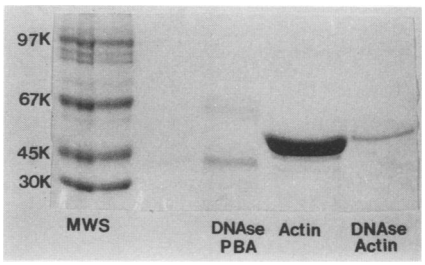

FIG. 2. SDS–5 to 15% polyacrylamide gradient gel of whole-cell streptococcal PBA extract eluted from a DNase I-agarose affinity column with 3 M guanidine-HCl (DNase-PBA). Purified rabbit actin was separated by SDS-PAGE (actin) as well as actin eluted from the same DNase I-agarose column with 3 M guanidine-HCl (DNase-actin). The blank lane shows that no significant PBA proteins eluted in the 0.75 M guanidine-HCl buffer. Protein size standards (lane MWS; rabbit muscle phosphorylase *b*, bovine serum albumin, egg albumin, and carbonic anhydrase) are shown (in kilodaltons).

powder was extracted for 30 min at 4°C in cold buffer A (2 mM Tris, 0.2 mM ATP, 0.5 mM 2-mercaptoethanol, 0.2 mM $CaCl_2$, and 0.005% sodium azide [pH 8.0]). The streptococcal cells were collected by centrifugation (10,000 × *g*, 10 min), and the supernatant was retained. The cell pellet was extracted a second time with buffer A. The cells were collected by centrifugation, and the extracts were combined.

To polymerize the bacterial actinlike proteins in the extract, it was adjusted to 50 mM KCl, 2 mM $MgCl_2$, and 5 mM ATP in buffer A for 2 h at 4°C. Solid KCl was slowly added to the polymerized extract to a final concentration of 0.6 M for a high-salt wash. The polymerized bacterial actinlike extract (PBA), which had now become highly viscous, was removed from solution by centrifugation at 112,000 × *g* for 2.25 h. The resulting clear pellet was washed with buffer A containing 50 mM KCl, 2 mM $MgCl_2$, and 5 mM ATP and collected by centrifugation at 112,000 × *g*. The pellet was reconstituted in buffer A alone, depolymerized by dialysis with buffer A, clarified by centrifugation (112,000 × *g*, 2.25 h), and repolymerized as described above. Finally, the PBA was removed from solution by centrifugation (112,000 × *g*, 2.25 h) and stored at 4°C. All buffers contained the protease inhibitors phenylmethylsulfonyl fluoride (1 mM; Sigma) and tosyl-lysyl-chloromethylketone (1 mM; Sigma). Rabbit skeletal actin, polymerized simultaneously for comparison with the bacterial extract, behaved identically to the PBA. Under these conditions for polymerization, actin is known to polymerize from G-actin or monomeric actin to a filamentous form, or F-actin (25).

Streptococcal proteins isolated and concentrated by polymerization were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in 5 to 15% polyacrylamide gradient gels by the method of Laemmli (17) as described previously (1). Proteins were separated by electrophoresis, subsequently transferred to nitrocellulose membranes, and reacted in immunoblots (33) with an antiactin MAb (Dupont NEN). Proteins in the PBA extract were visualized by Coomassie blue staining of a separate panel of the Western immunoblot (Fig. 1). Two proteins of about 60 kDa in the PBA reacted with the antiactin MAb. The data suggest that specific proteins in *S. pyogenes* resemble actin by their ability to polymerize or aggregate under the same conditions as eukaryotic actin and share immunological epitopes with human actin.

Since eukaryotic actin binds with high affinity to DNase I (18), the whole-cell PBA extract was examined for DNase I binding. DNase I-agarose beads (Worthington Biochemical Corp.) were mixed with PBA extract proteins overnight in column buffer (0.5 M sodium acetate, 1 mM $CaCl_2$, 30% glycerol [pH 6.5]). The beads were washed to remove unbound proteins and then eluted with 0.75 and 3.0 M guanidine-HCl column buffer in a stepwise fashion. No significant protein was eluted in the 0.75 M guanidine HCl

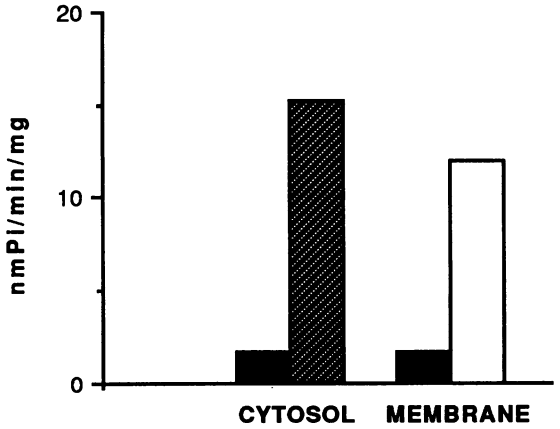

FIG. 3. Enhancement of myosin-ATPase activity by PBA isolated from the cytosol and membrane of *S. pyogenes* M⁻. Myosin alone (solid bars), myosin and streptococcal cytosol PBA (hatched bars), and myosin and streptococcal membrane PBA (open bars) were assayed for ATPase activity as described in Materials and Methods. The results are plotted as nanomoles of P_i per minute per milligram of protein. PBA alone was also tested at the same time, and the amount of P_i released or present was similar to that in the myosin-alone control (data not shown).

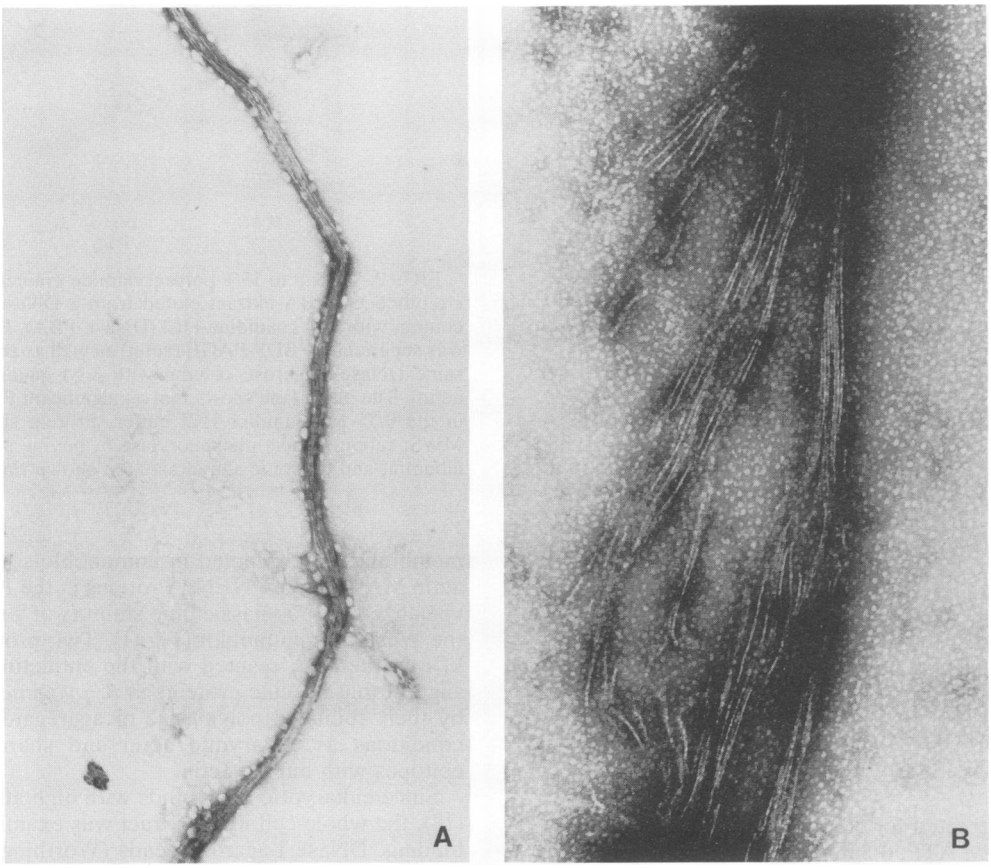

FIG. 4. Electron micrograph of negatively stained *S. pyogenes* PBA filaments (A) and rabbit skeletal actin filaments (B). Magnification, ×90,000.

buffer (blank lane, Fig. 2). Proteins eluted from the DNase column in 3 M guanidine-HCl were analyzed by SDS-PAGE (DNase-PBA lane, Fig. 2). The Coomassie blue-stained gels indicated two major PBA extract proteins of about 60 and 43 kDa eluted in the 3 M guanidine-HCl buffer (DNase-PBA, Fig. 2). There appeared to be more of the ~43-kDa than of the ~60-kDa protein. Since there was little evidence for a 43-kDa protein that reacted with the antiactin MAb, the data suggested that DNase binding of the protein might not necessarily correlate with recognition by specific antiactin antibody. Rabbit skeletal actin, used as a control, bound to DNase I under the same conditions (Fig. 2) and was eluted with 3 M guanidine-HCl buffer (DNase-actin, Fig. 2). Therefore, *S. pyogenes* proteins in the PBA extract of about 60 and 43 kDa behave like eukaryotic actin by binding to DNase I. The strong association of eukaryotic actin with DNase I is a consistent experimental finding (18). Although the function for the affiliation of actin with DNase is unknown, the data presented here suggest that specific streptococcal proteins share this unique activity with eukaryotic actin.

A biological function of eukaryotic actin is the enhancement of myosin-ATPase activity (14). To test PBA extracts for their ability to increase eukaryotic myosin-ATPase activity, PBA extracts were added separately to purified human cardiac myosin in the presence of ATP and Mg^{2+}. Eukaryotic myosin-ATPase activity was measured by Pollard's modification (26) of the method of Martin and Doty in an assay to determine the release of P_i from ATP. Human cardiac myosin (74 μg) and rabbit skeletal actin (15 μg) or PBA (15 μg) were incubated at 37°C for exactly 30 min in a total volume of 0.5 ml of reaction buffer. Enhancement of ATPase activity by PBA extracts from M^- *S. pyogenes* was determined by comparing the ATPase activity of myosin alone with the activity of the PBA extract combined with myosin. PBA extracts increased myosin ATPase activity approximately seven- to ninefold over the ATPase activity of myosin alone (Fig. 3). PBA extracts alone had activity approximately equal to that of myosin alone (data not shown). Rabbit skeletal muscle actin enhanced the myosin ATPase activity 9 to 10 times above the background level of myosin alone (data not shown).

Since the bacterial proteins are not completely purified and cannot be compared with moles of purified actin, it is not clear whether the bacterial actinlike proteins behave kinetically like eukaryotic actin in ATPase activation of myosin. However, enhancement of ATPase activity is clearly evident when PBA is added to human cardiac myosin. Although not shown, proteins of about 60 and 43 kDa bound to myosin affinity columns and were eluted with 3 M KCl. These proteins reacted with the antiactin MAb. These data are supported by earlier observations of actinlike proteins in other bacteria (22–24), in which proteins of 43 kDa were capable of binding to myosin. In these studies, actinlike proteins also had solubility characteristics and electrophoretic mobilities similar to those of vertebrate actin (22, 24).

Because polymerized eukaryotic actin filaments can be viewed by electron microscopy, whole-cell PBA was examined for the presence of filaments in this manner. Both rabbit

actin and PBA protein were diluted to 0.1 mg/ml and polymerized. Samples were immediately applied to carbon-coated copper grids and negatively stained with uranyl acetate as described previously (4). The proteins were viewed by electron microscopy to locate the filaments on the grids (Fig. 4). Individual filaments of rabbit actin were 10 nm wide (Fig. 4B), and the individual bacterial actinlike filaments were 2 to 4 nm wide (Fig. 4A) within the larger filament bundle. The bacterial proteins formed a much narrower filament than eukaryotic actin, suggesting that the bacterial proteins were quite different. Filaments in some cases were associated with bound proteins, which obscured parts of the filament. However, electron microscopy verified that streptococcal PBA-like proteins were able to polymerize into a filamentous form under conditions for polymerization of filamentous actin.

Since the origin of bacterial and streptococcal actinlike proteins is unknown, we hybridized the *Drosophila* actin gene to streptococcal DNA in Southern blots (data not shown) to determine whether nucleotide sequences that were homologous with eukaryotic actin were present in M^+ or M^- streptococcal DNA. However, under low-stringency conditions, no hybridization was observed in autoradiographs, while the actin gene probe reacted positively with itself. The data suggest that if there are actins in streptococci, their nucleotide sequence does not have homology with the *Drosophila* actin gene. Bacterial actinlike proteins may have structures and functions similar to those of eukaryotic actin although their nucleotide sequences are dissimilar.

Although the mechanism of actin and role of actins or actin-binding proteins in bacteria are unknown, models of contraction in nonmuscle eukaryotic cells have suggested that the attachment of actin to the cell membrane stabilizes the interaction of actin with myosin (31). More than 60 different proteins have been shown to bind to eukaryotic actin (27), including the M protein of *S. pyogenes* (3). It is possible that our PBA extract contains a family of interactive actinlike molecules. In eukaryotic cells, actin-binding proteins appear to play a major role in the regulation of the polymerization and depolymerization of actin (27). Because of the absolute requirement for actin in eukaryotic cells from the plant and animal kingdoms, the presence of an actinlike protein in streptococci is not surprising. It has been postulated that actinlike proteins in prokaryotes may function in cell division, motility, cell metabolism, and maintenance of cell shape (22, 24). The function of an actinlike protein(s) in *S. pyogenes* is currently under investigation.

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