An Ectoprotein Kinase of Group C Streptococci Binds Hyaluronan and Regulates Capsule Formation*

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A 56-kDa protein had been isolated and cloned from protoplast membranes of group C streptococci that had erroneously been identified as hyaluronan synthase. The function of this protein was reexamined. When streptococcal membranes were separated on an SDS-polyacrylamide gel and renatured, a 56-kDa protein was detected that had kinase activity for a casein substrate. When this recombinant protein was expressed in Escherichia coli and incubated in the presence of [32P]ATP, it was responsible for phosphorylation of two proteins with 30 and 56 kDa that were not present in the control lysate. The 56-kDa protein was specifically phosphorylated in an immunoprecipitate of a detergent extract of the recombinant E. coli lysate with antibodies against the 56-kDa protein, indicating that it was autophosphorylated. The E. coli lysate containing the recombinant protein could bind hyaluronan, and hyaluronan binding was abolished by the addition of ATP. Kinetic analysis of hyaluronan synthase and release from isolated protoplast membranes indicated that phosphorylation by ATP stimulated hyaluronan release and synthesis. Incubation of membranes with antibodies to the 56-kDa protein increased hyaluronan release. The addition of [32P]ATP to intact streptococci led to rapid phosphorylation of two proteins, 56 and 75 kDa each at threonine residues. This phosphorylation was neither observed with [32P]phosphate nor in the presence of trypsin, indicating that the kinase was localized extracellularly. The addition of ATP to growing group C streptococci led to increased hyaluronan synthesis and release. However marked differences were found between group A and group C streptococci. Antibodies against the 56-kDa protein from group C streptococci did not recognize proteins from group A strains, and a homologous DNA sequence could not be detected by polymerase chain reaction or Southern blotting. In addition, Group A streptococci did not retain a large hyaluronan capsule like group C strains. These results indicated that the 56-kDa protein is an ectoprotein kinase specific for group C streptococci that regulates hyaluronan capsule shedding by phosphorylation.

Group A and C streptococci are pathogens capable of causing a variety of infections. Group A streptococci are known to initiate postinfectious sequelae in humans such as acute rheumatic fever and glomerulonephritis. Group C streptococci are primarily animal pathogens. Many strains of both groups A and C streptococci are able to surround themselves with a hyaluronan capsule that has been implicated as a major virulence factor (1–5). Early attempts to clone the hyaluronan synthase from group C streptococci led to the erroneous identification of a 56-kDa protein (6, 7). The identification was based on indirect evidence, since synthase activity could not be reconstituted. This protein was affinity-labeled with the peridate-oxidized nucleotide sugars UDP-GlcNAc and UDP-glucuronic acid, and it bound to nascent hyaluronan. Binding of protoplast membrane proteins to nascent hyaluronan was used to develop a new method that extracted the enzyme activity together with two proteins of 42 and 56 kDa (8). Final purification of the synthase yielded the 42-kDa protein in active and electrophoretically homogeneous form. DeAngelis et al. (9–11) and Dougherty and van de Rijn (12) proved by genetic deletion analysis that the 42-kDa protein was the streptococcal hyaluronan synthase. Therefore the function of the 56-kDa proteins was reexamined. Its amino acid sequence contained an ATP binding domain and indicated homology to bacterial transport proteins and several hyaluronan binding sites (7). It had an N-terminal signal sequence that could integrate it into protoplast membranes. The 56-kDa protein was processed to a 54-kDa protein by endogenous proteases and shed into the culture medium (13). This protein also elicited antibodies in patients with rheumatic fever and cross-reacted with surface protein form eukaryotic cells (14). The cross-reacting eukaryotic protein had a molecular mass of 52 kDa and formed a complex with the hyaluronan receptor RHAMM (15). In this publication we showed that the 56-kDa protein from group C streptococci is an extracellular hyaluronan-binding protein that has a threonine kinase activity and regulates hyaluronan capsule formation.

EXPERIMENTAL PROCEDURES

Materials—Group C Streptococcus equisimilis (strain D181) was obtained from the Rockefeller University collection; other streptococcal strains were received from the culture collection of Dr. Wagner, University of Jena, Germany. Radiochemicals were purchased from Amer sham Pharmacia Biotech, and other reagents were from Sigma.

General Methods—The S. equisimilis strain D181 was grown in Hewitt-Todd medium, and protoplast membranes were prepared as described (7). Proteins were separated by electrophoresis on 10% SDS-polyacrylamide gel and renatured, a 56-kDa protein was detected that had kinase activity for a casein substrate. These article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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In some experiments the 100 µg of membranes were dephosphorylated with 2 units of alkaline phosphatase (Boehringer Mannheim) in 50 µl of 50 mM Tris-HCl, 0.1 mM EDTA, pH 8.5, for 1 h at 37 °C and recovered by ultracentrifugation in an Airfuge (Beckman) at 100,000 × g for 30 min. Controls were incubated similarly without alkaline phosphatase. Aliquots of 50 µl were mixed at 27 °C with 0.5 µCi of [32P]ATP, 10 mM MgCl₂ for 10 min at 37 °C. Bacteria were sedimented by centrifugation for 15 min at 10,000 × g and resuspended in 40 µl of prewarmed Todd-Hewitt medium containing 4 mM MgCl₂. Aliquots of 4 ml were incubated with 200 µl of [32P]phosphate (specific activity 10 mCi/µl) or with 200 µl of [γ-32P]ATP (specific activity, 10 mCi/ml) in the absence and presence of 160 units of the extracellular adenosine 5′-triphosphatase apyrase. Aliquots of 1 ml were withdrawn after 5 and 15 min and centrifuged at 10,000 × g for 1 min. The bacterial sediments were frozen until further analysis. The samples were resuspended in sample buffer and subjected to gel electrophoresis on 10% SDS-polyacrylamide.

Radioactivity was visualized by autoradiography.

Inhibition of phosphorylation by extracellular trypsin was determined by incubation of 3 ml of the above bacterial suspension with 30 µCi of [γ-32P]ATP in the presence of 0.005% trypsin for 15 min at 37 °C. Phosphorylation was visualized as described above.

Phosphorylation of extracellular casein by growing streptococci was determined by incubating 3 ml of the above bacterial suspension with 30 µCi of [γ-32P]ATP in the presence of 0.1, 1, and 100 µg of casein for 15 min at 37 °C. Bacteria were sedimented by centrifugation for 5 min at 10,000 × g, proteins in the culture media were precipitated by the method of Wessel and Flugge (21) and separated by gel electrophoresis on 10% SDS-polyacrylamide, and radioactivity was visualized by autoradiography.

Adsortion of Radioactive Hyaluronan to the Recombinant 56-kDa Protein—Radioactive hyaluronan was prepared by incubating 150 µg of streptococcal membranes at 37 °C with 500 µl of a solution of 160 µCi UDP-GlcNac and 8 µCi UDP-1⁴Cglucuronic acid (specific activity 320 mCi/mmol), 1 mM diithiothreitol, 10 mM MgCl₂ for 2 h. Proteins were denatured by heating for 5 min to 100 °C, and the solution was clarified by centrifugation for 5 min at 14,000 × g. The supernatant was dialyzed against water and used for adsorption with E. coli lysates.

After cell lysis of E. coli Y1909 lysed by growth of Agt 11 or Agt 11/2LK, the debris were sedimented at 3,000 × g for 10 min, and the supernatants were centrifuged at 100,000 × g for 30 min. The pellets were resuspended in 50 mM Tris-HCl, 10 mM MgCl₂, pH 7.0, at a concentration of 3 mg/ml, and 100 µl of a 1:1000 dilution of anti-56-kDa protein in blocking solution for 1 h. The filters were washed with phosphate-buffered saline and developed with anti-rabbit-IgG alkaline phosphatase conjugate as described (20).

Identification of the Kinase—Streptococcal membranes were separated on a SDS-polyacrylamide gel that contained casein as kinase substrate and tested for a kinase activity. After electrophoresis, proteins were reconstituted, and the gel was incubated with γ-32P]ATP. The radioactive substrate was washed out, and the gel was autoradiographed. One band was visualized with a molecular mass of 56 kDa (Fig. 1).

The recombinant 56-kDa protein was expressed also in E. coli and analyzed for kinase activity. Membranes isolated from E. coli Agt-11 and E. coli Agt-11/2LK lysates were incubated with [γ-32P]ATP, and the amount of radioactivity incorporated into proteins was determined to be 3916 cpm/µg for normal and 5781 cpm/µg for recombinant cells. After SDS-polyacrylamide gel electrophoresis, the pattern of phosphorylated proteins was compared. Fig. 2 shows that both lysates contained phosphorylated proteins. However, the E. coli Agt-11/2LK lyase containing the cloned 56-kDa protein showed a second additional phosphorylated band in lanes with 16 and 30 kDa. Antibodies against the 56-kDa protein were used for immunoprecipitation of the 56-kDa protein from a detergent extract of the particular fraction from an E. coli Agt-11/2LK lyase. The antiserum has been shown to react specifically with the 56-kDa protein in Western blots from membranes of the streptococcal strain D181 and recombinant E. coli Agt-11/2LK.
also immunoprecipitated from a detergent extract of 32P-labeled membranes. This protein was amino acid, and X was any other amino acid (22). It was also

lysates (7). The immunoprecipitate was incubated with [γ-32P]ATP, and the phosphorylated proteins were analyzed. Fig. 2 (lane C) shows that the recombinant 56-kDa protein was phosphorylated in the immunoprecipitate. This protein was also immunoprecipitated from a detergent extract of 32P-labeled membranes (data not shown). An immunoprecipitate from a control lysate of E. coli lacking the recombinant 56-kDa protein with antisera against the 56-kDa protein (C), incubated with [γ-32P]ATP, and analyzed as above.

Previously, a membrane-bound kinase of the Hpr system has been described in streptococci that was involved in sugar transport (18). Therefore, we tested whether the 56-kDa protein was able to phosphorylate the phosphate carrier protein Hpr. An E. coli lysate containing the active recombinant 56-kDa kinase did not phosphorylate the streptococcal Hpr protein (data not shown).

Hyaluronan Binding Activity in Recombinant 56-kDa Protein—The 56-kDa protein contains several hyaluronan binding motifs (7) that are characterized by BX2BX, where B is a basic amino acid, and X is any other amino acid (22). It was also extracted together with the hyaluronan synthase from streptococcal membranes by a recently developed procedure that enriched only hyaluronan-binding membrane proteins (8). We therefore examined whether the hyaluronan binding activity was retained by the recombinant protein expressed in E. coli. E. coli lysates were prepared from λgt-11/2LK and λgt-11-control phages. Membranes were prepared by sedimentation in the ultracentrifuge. The membranes were incubated in the absence and presence of 1 mM ATP with radioactive hyaluronan to analyze the possibility that hyaluronan binding was influenced by phosphorylation. The membranes were again sedimented by ultracentrifugation, and the radioactivity in the membrane pellet was determined. Fig. 3 shows that the lysate containing recombinant 56-kDa protein bound hyaluronan only in the absence of ATP.

Kinetics of Hyaluronan Synthesis and Release—The kinetics of hyaluronan synthesis and release was measured in the presence and absence of ATP. Fig. 4 shows that hyaluronan is synthesized at membranes and then released into the soluble fraction. Hyaluronan release ceased after 60 min in the absence of ATP. In the presence of ATP, membranes continued to release hyaluronan into the soluble fraction.

Attempts to delete the 56-kDa kinase by homologous recombination were not successful, suggesting that it plays a critical, nonredundant role in the cell. Therefore specific antibodies were utilized to verify its influence on hyaluronan synthesis. The kinetics of hyaluronan synthesis and release was measured before and after binding of antibodies to streptococcal membranes. Fig. 5 shows that antibodies to the 56-kDa protein increased the rate of hyaluronan release from membranes into the supernatant.

Extracellular Localization and Strain Specificity of the 56-kDa Protein—The following experiments should clarify the cellular localization and strain specificity of the 56-kDa protein. Bacterial colonies on agar plates containing hyaluronidase were overlaid with nitrocellulose filters to produce replicates. The filters were developed with polyclonal rabbit antisera against the 56-kDa protein and anti-rabbit peroxidase. Group C streptococci (15 strains) and group A streptococci (18 strains) were tested. Positive signals were obtained from all group C streptococci but not from group A streptococci (data not shown). When protoplast membranes were prepared from different strains and analyzed by Western blotting with antisera against the 56-kDa protein, the 56-kDa protein could again be
detected only in membranes from group C streptococci but not in protoplast membranes form group A streptococci. Furthermore it was impossible with polymerase chain reaction and southern hybridizations at low stringency to identify the homologous gene in group A streptococci.

For extracellular localization of the kinase, intact group C streptococci were incubated with \(^{32P}\)phosphate or \(^{32P}\)ATP for various periods, and phosphorylation was followed by polyacrylamide gel electrophoresis and autoradiography (Fig. 6) in the presence and absence of adenosine 5'-triphosphatase. Two proteins with molecular masses of 56 and 75 kDa were phosphorylated only by extracellular ATP after 5 and 15 min in the absence of adenosine 5'-triphosphatase. Longer incubation times did not yield labeled proteins, indicating that phosphorylation was transient and susceptible to endogenous surface phosphatases. Neither extracellular \(^{32P}\)phosphate that could be taken up by bacteria nor \(^{32P}\)ATP in the presence of adenosine 5'-triphosphatase led to phosphorylation, excluding the possibility that intracellular \(^{32P}\)phosphate caused the phosphorylation. Low concentrations of extracellular trypsin eliminated phosphorylation. Extracellular casein was also a target of the kinase (Fig. 6).

When membranes were labeled for 15 min and chased with unlabeled ATP for 15 min, the 56-kDa protein disappeared and gave rise to a 36-kDa protein (data not shown), suggesting that the 56-kDa protein was rapidly degraded. The radioactive proteins were eluted from the gel and subjected to phosphoamino acid analysis. The phosphorylated amino acid was threonine (Fig. 7).

**Extracellular ATP Decreases Capsule Formation and Increases Hyaluronan Production**—The influence of the ectoprotein kinase and extracellular ATP on the hyaluronan synthesis and shedding was investigated on growing cultures of group C and group A streptococci. Increasing concentrations of ATP were added to group C streptococcal strain D181. The bacteria were separated from the culture medium, and the amount of hyaluronan on bacteria and in the medium was determined (Fig. 8). ATP reduced the amount of hyaluronan on bacteria and increased the concentration in the culture medium. This led to an overall increase in hyaluronan synthesis. Treatment with alkaline phosphatase decreased the amount of hyaluronan in the medium (Table I). In comparison, group A streptococcal strain 36487 released most of its hyaluronan capsule into the medium also in the absence of ATP, and alkaline phosphatase reduced the amount of hyaluronan in the medium only slightly. Treatment of group C streptococci with proteinase E led to an increased shedding of hyaluronan into the culture medium, whereas a cysteine proteinase inhibitor showed only marginal effects. Despite the differences in hyaluronan capsule between group A and group C streptococci, the macroscopic appearance of colonies on agar plates were mucoid with both strains.

Because an ectoprotein kinase was found to be involved in growth regulation of fibroblasts (23), we measured the effect of increasing ATP concentrations on the growth rate of group A and group C streptococci. Extracellular ATP concentrations above 10 mM had only slight growth inhibitory effects.

**DISCUSSION**

In previous publications we reported cloning of a 56-kDa protein from protoplast membranes of group C streptococci that was erroneously characterized as the hyaluronan synthase (6, 7). Here we have reexamined the function of this protein that bound hyaluronan (8) and exhibited sequence similarity with ATP-binding transport proteins (7). We show that a protein from streptococcal membranes with a molecular mass of 56 kDa possessed a protein kinase activity that was also retained when the protein was expressed in *E. coli*. The recombinant 56-kDa protein could be autophosphorylated in an immunoprecipitate. The kinase was not identical to the previously identified streptococcal Hpr kinase of the sugar transport system (18). The 56-kDa protein and its gene was only detected in group C streptococci but not in group A streptococci. In streptococcal membranes, the kinase activity was directed against a 75-kDa and the 56-kDa membrane proteins. The function of the 75-kDa protein phosphorylation remains elusive, but it is conceivable that it mediates transduction of other signals in response to extracellular ATP.

The localization of the 56-kDa kinase was investigated by the kinetics of phosphorylation with exogenous \(^{32P}\)phosphate and \(^{32P}\)ATP of intact growing bacteria. Rapid phosphorylation was only observed with \(^{32P}\)ATP and was abolished by extracellular trypsin. Extracellular casein could also serve as a substrate. To our knowledge this is the first demonstration of an ectoprotein kinase in prokaryotes, although there are several examples of eukaryotic ectoprotein kinases that mediate signal transfer from extracellular ATP into the cell (23–29).

In addition to the kinase activity, a hyaluronan binding activity could be demonstrated in recombinant *E. coli* lysates that bound exogenous hyaluronan only in the absence of ATP, suggesting that hyaluronan binding could be influenced by autophosphorylation. Thus, extracellular ATP influenced the kinetics of hyaluronan synthesis and release from isolated streptococcal protoplast membranes. Membranes continued to release hyaluronan in the presence of ATP but stopped this process after 60 min in its absence. Antibodies against the 56-kDa protein also increased the shedding rate of hyaluronan from membranes.

Extracellular ATP at low millimolar concentrations stimulated hyaluronan synthesis and shedding from growing group C streptococci but not in group A streptococci. In the infected host, extracellular ATP can be released from inflamed neutrophils or degranulated platelets (30). The effective ATP concentrations seem to be physiological, because they were slightly below the intracellular concentration that has been measured to be about 6 mM (31). Extracellular phosphatase reduced and extracellular proteinase increased hyaluronan shedding rate. These processes were only slightly influenced by an inhibitor of the extracellular cysteine proteinase that was shown to con-
Fig. 5. Influence of anti-56-kDa protein antibodies on the kinetics of hyaluronan synthesis and release. Streptococcal protoplast membranes (5 mg in 5 ml of phosphate-buffered saline) were incubated with 0.5 ml of preimmune serum or with antiseraum against the 56-kDa protein for 24 h at 4 °C and reisolated by ultracentrifugation. The kinetics of hyaluronan synthesis and release were determined as described under “Experimental Procedures.” O, preimmune serum; ●, anti-56 protein; —, membrane-bound; --, supernatant. The error bars indicate the S.D. of triplicate samples (p value for the difference at 24 h, 0.05).

Fig. 6. Extracellular phosphorylation. Whole streptococci (strain D181) were incubated with [32P]phosphate (P) or [32P]ATP for 5 and 15 min in the presence and absence of adenosine 5′-triphosphatase or with increasing concentrations of casein or with trypsin. Phosphorylated proteins were separated by polyacrylamide gel electrophoresis and visualized by autoradiography as described under “Experimental Procedures.”

Fig. 7. Phosphoamino acid analysis. The [32P]-labeled 56-kDa protein was eluted and subjected to phosphoamino acid analysis as described under “Experimental Procedures.” P-, phospho-

Fig. 8. Stimulation of hyaluronan synthesis and release by extracellular ATP. Streptococci (strain D181) were grown to A600 = 0.6 in the presence of increasing concentrations of ATP. The amount of hyaluronan on the bacterial sediment (●) and in the culture supernatant (○) was determined as described under “Experimental Procedures.” □, total hyaluronan.

Table 1

| Treatment       | Streptococcus group C (strain D181) | Streptococcus group A (strain 36487) |
|-----------------|------------------------------------|-------------------------------------|
|                 | Capsule at A600 = 0.6               | Capsule at A600 = 0.6               |
| Control         | 418                                | 22                                  |
| + Phosphatase   | 416                                | 25                                  |
| + Proteinase E  | 126                                | 30                                  |
| + Proteinase inhibitor | 360                            | 28                                  |

Contribution to the virulence of group A streptococci (32). Nevertheless, it is possible that other proteases participate in regulation of capsule formation.

Van de Rijn has shown that the hyaluronan capsule is lost during the stationary phase (33). In addition, we demonstrated here that group C streptococci, but not group A streptococci, regulate their capsule in response to extracellular ATP during the growth phase. The differences in hyaluronan synthesis between group A and group C streptococci may be a reflection of different host specificity. Retention of a larger hyaluronan capsule by group C streptococci in skin surfaces may be more advantageous for their survival in preventing desiccation. Invasion into necrotic host tissue would expose them to host defense that calls for rapid shedding of surface hyaluronan together with adhered antibodies or host cells. Thus the 56-kDa ectoprotein kinase may contribute to the virulence of group C streptococci.

Our results also showed that binding of nascent hyaluronan by a cell surface receptor did not only inhibit hyaluronan release but also hyaluronan synthase activity in isolated membranes and hyaluronan synthesis in whole cells. This finding may be a reflection of a novel mechanism for the regulation of
polymer biosynthesis. It appears that chain initiation or elongation is suppressed if dissociation of nascent hyaluronan from the synthase into the medium is inhibited by cell surface receptors. This mechanism may also apply for hyaluronan synthesis in eukaryotic cells that could be influenced by cell surface receptors such as CD44 or RHAMM.

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