Induction of Group A *Streptococcus* Virulence by a Human Antimicrobial Peptide

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Group A *Streptococcus* (*Streptococcus pyogenes* or GAS) is the agent of streptococcal pharyngitis, one of the most common childhood bacterial infections, as well as infections of the skin and soft tissues and less common but potentially life-threatening syndromes such as necrotizing fasciitis and streptococcal toxic shock. It is responsible, as well, for the postinfectious syndromes of post-streptococcal glomerulonephritis and acute rheumatic fever, a leading cause of acquired heart disease in many developing countries.

GAS produce a variety of surface molecules and secreted products that contribute to pathogenesis. Among these, two factors have been shown most convincingly to play a central role in virulence: the major surface antigen, M protein, and the GAS capsular polysaccharide, which is composed of hyaluronic acid. Certain strains of GAS produce large amounts of hyaluronic acid capsule giving a mucoid appearance to their colonies on blood agar. The fact that mucoid strains of GAS have been associated with both invasive infection and acute rheumatic fever has suggested a role for the capsular polysaccharide in virulence. The occurrence of outbreaks of acute rheumatic fever associated with mucoid strains of GAS at several locations in the United States in the 1980s supported the same inference. These clinical and epidemiologic observations implicating the capsule in pathogenesis have been corroborated by extensive experimental studies that have demonstrated a definite role of the hyaluronic acid capsule as a virulence factor in GAS infection.

Direct evidence that the capsule contributes to virulence has come from several studies using acapsular mutant strains of GAS derived by transposon mutagenesis or by targeted inactivation of the gene(s) required for hyaluronic acid synthesis. Because the capsule is required for GAS resistance to complement-mediated phagocytic killing, it is not surprising that capsule expression enhances virulence in systemic infection models in which the animals succumb to overwhelming bacteremia. In addition, however, the hyaluronic acid capsule contributes in a pivotal fashion to the capacity of the organisms to produce invasive soft tissue infection. A mouse model of invasive GAS soft tissue infection has been developed in which the bacterial inoculum is delivered by superficial injection just below the skin surface, in a small volume, and with minimal trauma to the tissues (1, 2). Mice challenged with a highly encapsulated M24 strain developed dermal necrosis with underlying purulent inflammation and secondary bacteremia, while mice challenged with an acapsular transposon mutant strain developed no lesion at all or minor superficial inflammation and no bacteremia. Similar results were observed in the same model with a moderately encapsulated M3 strain of GAS (originally isolated from a patient with necrotizing fasciitis) that was compared with two isogenic acapsular mutants derived by allelic exchange mutagenesis of the *hasA* (hyaluronate synthase) gene. The dramatic impact of the capsule on virulence in this model is correlated with equally striking histopathologic findings. In animals challenged with an acapsular mutant, a small focus of neutrophilic inflammation and necrosis is confined by a well-formed abscess. By contrast, after challenge with the encapsulated wild-type strain, acute inflammatory cells extend throughout the subcutaneous tissue and are associated with thrombosis of blood vessels, with infarction and necrosis of the overlying dermis, a histopathologic picture very similar to that in patients with GAS necrotizing fasciitis. Examination of the histologic sections by immunofluorescence microscopy with a GAS-specific antibody showed that the acapsular GAS cells were confined to the abscess cavity, while the encapsulated wild-type organisms were widely dispersed throughout the subcutaneous tissues.

The GAS capsular polysaccharide is composed of hyaluronic acid, a high molecular weight linear polymer made up of β (1→4)-linked disaccharide repeat units of d-glucuronic acid (1→3)-β-d-*N*-acetylglucosamine. The polysaccharide is synthesized from

the nucleotide sugar precursors UDP-glucuronic acid and UDP-*N*-acetylglucosamine by a membrane-associated hyaluronate synthase. A genetic locus required for hyaluronic acid production in GAS consists of a cluster of three genes, *hasABC,* whose products are hyaluronate synthase and two other enzymes required for hyaluronic acid biosynthesis (3-5). The simplicity of the capsule gene cluster in GAS contrasts with the size and complexity of capsule synthesis loci in other encapsulated gram-positive bacteria, which generally consist of at least 12 genes.

The *has* operon is highly conserved among GAS strains. Despite the high degree of conservation of the hyaluronate synthase gene, individual GAS strains vary widely in the amount of capsule they produce. Since the *has* genes are conserved, differences in capsule expression among strains are likely to reflect differences in the regulation of *has* gene transcription. Capsule production varies not only among strains but also in an individual strain under different circumstances. The dynamic changes in GAS capsule expression with changes in growth rate and the observation that some strains of GAS appear more highly encapsulated after animal passage or when freshly isolated from patients have suggested that capsule gene transcription is regulated by cellular mechanisms in addition to those attributable to the intrinsic structure of the *has* operon promoter.

Transposon mutagenesis of a poorly encapsulated M3 strain of GAS resulted in the identification of a novel regulatory locus whose inactivation dramatically increased capsule production (6). The locus consists of two genes, *csrR* and *csrS,* whose predicted products resemble the regulator and sensor proteins, respectively, of bacterial two-component regulatory systems. Targeted inactivation of *csrR* by allelic exchange mutagenesis resulted in a six-fold increase in capsule production. The increase in hyaluronic acid synthesis was accompanied by a parallel increase in *has* gene transcription, evidence that CsrR regulates transcription of the capsule synthesis genes. Subsequent work in several laboratories has demonstrated that the CsrRS system (also known as CovRS) influences expression of more than 100 genes, including those encoding a number of virulence determinants in addition to the hyaluronic acid biosynthetic locus (7-10).

Studies from our laboratory demonstrated that environmental Mg^{2+} concentration is a potent and specific stimulus for CsrR/CsrS-mediated regulation (11, 12). We studied the effect of divalent cations on expression of the Csr-regulated hyaluronic acid capsule genes (*hasABC*) by measuring chloramphenicol acetyltransferase (CAT) activity in a reporter strain of GAS carrying a *has* operon promoter-*cat* fusion. Addition of Mg^{2+}, but not of Ca^{2+}, Mn^{2+}, or Zn^{2+}, repressed capsule gene expression by up to 80% in a dose-dependent fashion. The decrease in capsule gene transcription was associated with a marked reduction in cell-associated capsular polysaccharide. RNA hybridization analysis demonstrated reduced expression of the Csr-regulated *hasABC* operon, streptokinase (*ska*), and streptolysin S (*sagA*) during growth in the presence of 15 mM Mg^{2+} for the wild-type strain 003CAT but not for an isogenic *csrS* mutant. We propose that Mg^{2+} binds to CsrS to induce phosphorylation of CsrR and subsequent repression of virulence gene expression. The low concentration of Mg^{2+} in extracellular body fluids predicts that the CsrR/CsrS system is maintained in a relatively inactive state during infection, thereby allowing increased expression of critical virulence determinants in the human host.

More recently, we have shown that subinhibitory concentrations of the human antimicrobial cathelicidin peptide LL-37 stimulate expression of the GAS capsule synthesis operon (*hasABC*) (13). Up-regulation is mediated by the CsrRS two-component regulatory system: it requires a functional CsrS sensor protein and can be antagonized by increased extracellular Mg^{2+}, the other identified environmental signal for CsrS. Up-regulation was also evident for other CsrRS-regulated virulence genes, including the IL-8 protease PrtS/ScpC and the integrin-like/IgG protease Mac/IdeS, findings that suggest a coordinated GAS virulence response elicited by this antimicrobial immune effector peptide. LL-37 signaling through CsrRS led to a marked increase in GAS resistance to opsonophagocytic killing by human leukocytes, an in vitro measure of enhanced GAS virulence, consistent with increased expression of the antiphagocytic capsular polysaccharide and Mac/IdeS. We propose that the human cathelicidin LL-37 has the paradoxical effect of stimulating CsrRS-regulated virulence gene expression, thereby enhancing GAS pathogenicity during infection. The ability of GAS to sense and respond to LL-37 may explain, at least in part, the unique susceptibility of the human species to streptococcal infection.

References

1) Ashbaugh CD, Warren HB, Carey VJ and Wessels MR. Molecular analysis of the role of the group A streptococcal cysteine protease, hyaluronic acid capsule, and M protein in a murine model of human invasive soft-tissue infection. *J. Clin. Invest* 1998;102:

550-560.
2) Schrager HM, Rheinwald JG and Wessels MR. Hyaluronic acid capsule and the role of streptococcal entry into keratinocytes in invasive skin infection. *J. Clin. Invest* 1996;98:1954-1958.
3) Crater DL, Dougherty BA and van de Rijn I. Molecular characterization of *hasC* from an operon required for hyaluronic acid synthesis in group A streptococci. *J. Biol. Chem* 1995;270: 28676-28680.
4) DeAngelis PL, Papaconstantinou J and Weigel PH. Molecular cloning, identification, and sequence of the hyaluronan synthase gene from group A *Streptococcus pyogenes*. *J. Biol. Chem* 1993; 268:19181-19184.
5) Dougherty BA, van de Rijn I. Molecular characterization of *hasB* from an operon required for hyaluronic acid synthesis in group A streptococci. *J. Biol. Chem* 1993;10:7118-7124.
6) Levin JC, Wessels MR. Identification of *csrR/csrS*, a genetic locus that regulates hyaluronic acid capsule synthesis in group A *Streptococcus*. *Mol. Microbiol* 1998;30:209-219.
7) Dalton TL, Collins JT, Barnett TC and Scott JR. RscA, a member of the MDR1 family of transporters, is repressed by CovR and required for growth of Streptococcus pyogenes under heat stress. *J Bacteriol* 2006;188:77-85.
8) Federle MJ, McIver KS and Scott JR. A response regulator that represses transcription of several virulence operons in the group A streptococcus. *J Bacteriol* 1999;181:3649-57.
9) Graham MR, Smoot LM, Migliaccio CA, et al. Virulence control in group A Streptococcus by a two-component gene regulatory system: Global expression profiling and in vivo infection modeling. *Proc Natl Acad Sci USA* 2002;99:13855-60.
10) Heath A, DiRita VJ, Barg NL and Engleberg NC. A two-component regulatory system, CsrR-CsrS, represses expression of three Streptococcus pyogenes virulence factors, hyaluronic acid capsule, streptolysin S, and pyrogenic exotoxin B. *Infect Immun* 1999;67: 5298-305.
11) Gryllos I, Grifantini R, Colaprico A, et al. Mg(2+) signalling defines the group A streptococcal CsrRS (CovRS) regulon. *Mol Microbiol* 2007;65:671-83.
12) Gryllos I, Levin JC and Wessels MR. The CsrR/CsrS two-component system of group A Streptococcus responds to environmental Mg2+. *Proc Natl Acad Sci USA* 2003;100:4227-32.
13) Gryllos I, Tran-Winkler HJ, Cheng MF, et al. Induction of group A Streptococcus virulence by a human antimicrobial peptide. *Proc Natl Acad Sci USA* 2008;105:16755-60.