Expression and Characterization of Group A Streptococcus Extracellular Cysteine Protease Recombinant Mutant Proteins and Documentation of Seroconversion during Human Invasive Disease Episodes

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A recent study with isogenic strains constructed by recombinant DNA strategies unambiguously documented that a highly conserved extracellular cysteine protease expressed by Streptococcus pyogenes (group A Streptococcus [GAS]) is a critical virulence factor in a mouse model of invasive disease (S. Lukomski, S. Sreevatsan, C. Amberg, W. Reichardt, M. Woischnik, A. Podbielski, and J. M. Musser, J. Clin. Invest. 99:2574–2580, 1997). To facilitate further investigations of the streptococcal cysteine protease, recombinant proteins composed of a 40-kDa zymogen containing a C192S amino acid substitution that ablates enzymatic activity, a 28-kDa mature protein with the C192S replacement, and a 12-kDa propeptide were purified from Escherichia coli containing His tag expression vectors. The recombinant C192S zymogen retained apparently normal structural integrity, as assessed by the ability of purified wild-type streptococcal cysteine protease to process the 40-kDa molecule to the 28-kDa mature form. All three recombinant purified proteins retained immunologic reactivity with polyclonal and monoclonal antibodies. Humans with a diverse range of invasive disease episodes (erysipelas, cellulitis, pneumonia, bacteremia, septic arthritis, streptococcal toxic shock syndrome, and necrotizing fasciitis) caused by six distinct M types of GAS seroconverted to the streptococcal cysteine protease. These results demonstrate that this GAS protein is expressed in vivo during the course of human infections and thereby provide additional evidence that the cysteine protease participates in host-pathogen interactions in some patients.

Virtually all strains of the Gram-positive pathogenic bacterium Streptococcus pyogenes (group A Streptococcus [GAS]) produce a highly conserved extracellular cysteine protease known as streptococcal pyrogenic exotoxin B (SpeB) (reviewed in reference 20). This enzyme is initially expressed as a 40-kDa zymogen, which is subsequently converted to a 28-kDa active protease (3–7, 16, 17, 26). Although the exact molecular basis whereby the zymogen-to-protease transformation occurs is unknown, evidence has been presented that the process can occur by autocatalytic truncation (16).

Several lines of evidence suggest that the cysteine protease or its zymogen is a GAS virulence factor in some patients (1, 2, 11–15, 18–21, 25). The enzyme degrades human fibronectin and vitronectin (15), two proteins involved in maintaining the integrity of the extracellular matrix and cell-cell interactions, and activates interleukin-1β precursor to biologically active interleukin-1β (11). It causes a rapid and destructive cytopathic effect when incubated with cultured human endothelial cells (15). It also activates a 66-kDa human matrix metalloprotease, a process that results in increased type IV collagenase activity (3). Herwald et al. (11) recently showed that the protease also directly releases biologically active kinins from their purified precursor protein, H-kininogen, in vitro, and from kininogens present in human plasma, ex vivo. Moreover, injection of the purified cysteine protease into the peritoneal cavity of mice causes progressive cleavage of plasma kininogens and kinin release (11).

Taken together, these and other data (20, 27) support the idea that the cysteine protease participates in host-pathogen interactions and detrimentally affects host physiologic processes in some patients with GAS disease. Recently, it has been documented that insertional inactivation of speB profoundly decreases the ability of GAS to kill mice after intraperitoneal injection (19). These studies, plus the observations that patients with invasive disease who have low levels of acute-phase serum antibody to the cysteine protease are more likely to die (12) and that immunization of mice with the enzyme confers protection against intraperitoneal challenge (13), indicate that additional studies of SpeB are warranted.

In this investigation, we analyzed zymogen processing by use of a site-specific mutant zymogen lacking autocatalytic truncation ability and wild-type 28-kDa active cysteine protease (22). We also assessed if patients with invasive streptococcal disease mount a serologic response to SpeB. Enzymatically active protease cleaves the mutant enzyme to form the 28-kDa protein. Patients with invasive episodes caused by strains expressing several M-protein serotypes seroconvert to SpeB, indicating that the molecule is made in vivo during the course of human invasive episodes.

MATERIALS AND METHODS

Bacterial strains and plasmids. The His tag expression vector pPROEX-1 (Gibco-BRL/Life Technologies, Grand Island, N. Y.) was used to construct plasmids for production of recombinant proteins. pSEBC2S is a derivative of plasmid...
plasmid (pGM-1) had an 81-bp segment encoding the 27-amino-acid secretion signal sequence, a vector that contained speB lacking this leader sequence was constructed. To construct this vector (pGM-5), the speB gene lacking the unwanted 81-bp region was amplified from pGM-1 and cloned with primers SG13 (5'-GGGG GCGGCCCATCACAACTTTGCTGTCG-3') and SG2 (Fig. 1). The amplified fragment was cloned into pPROEX-1, and ampicillin-resistant colonies were screened by PCR for the presence of speB. A colony that had speB in the correct orientation was identified. Automated DNA sequence analysis confirmed the correct reading frame and lack of spurious mutations.

Analogue strategies were used to construct plasmids with fragments of the speb gene containing codons 146 to 398 (pGM-8) and 28 to 145 (pGM-6). pGM-6 is designed to express a 28-kDa protein corresponding to the mature form of SpeB, and pGM-6 should express a 12-kDa molecule corresponding to the SpeB propeptide. pGM-6 was constructed from a speB PCR product generated with primers SG13 and SG14 (5'-CGTAGGGCGCTTATTTAATCTCAGCGTGA-3') and primers SG15 (5'-GGGGGGCGCCCAACCATGTTGTGATAACTC TCTCT-3') and SG2 were used to make the amplification product used for pGM-8 (Fig. 1). All DNA inserts were sequenced in their entirety to confirm that no undesired mutations were present.

Expression and purification of recombinant proteins. Recombinant proteins were obtained from E. coli BL21 containing the appropriate plasmids after induction with isopropyl-β-D-thiogalactopyranoside overnight at 30°C. Cultures were diluted 1:100 in Luria-Bertani medium supplemented with 100 μg of ampicillin per ml, and cultured to an optical density at 590 nm of 0.5 to 1.0. IPTG was added (final concentration, 0.6 mM) and the cells were grown for an additional 2 to 6 h. After centrifugation at 10,000 × g for 10 min, the supernatant was discarded and the cell pellet was stored at −70°C. The cells were resuspended in 50 mmol of Tris-HCl (pH 8.5) containing 10 mmol 2-mercaptoethanol and 1 mmol phenylmethylsulfonyl fluoride and lysed by sonication with three 20-s pulses generated by a Sonifier Cell Disrupter 250 (Branson, Danbury, Conn.). After each 20-s burst, the cells were cooled for 5 min in a dry-ice bath. Cell wall debris was removed by centrifugation at 100,000 × g for 10 min, and the supernatant was applied to a 1- to 5-ml column of Ni-nitrotriacetate acid resin (Qiagen, Chatsworth, Calif.) equilibrated with 50 mmol of Tris-HCl (pH 8.5), 100 mmol KCl, 10 mmol 2-mercaptoethanol, 10% (vol/vol) glycerol. The column was washed with 10 volumes of buffer A, 2 volumes of buffer B (20 mmol Tris-HCl [pH 8.5], 1 M KCl, 10 mmol 2-mercaptoethanol, 10% [vol/vol] glycerol) and 1% (vol/vol) glycerol to remove unbound protein. Recombinant protein containing the His tag was eluted from the column by washing with 5 volumes of buffer C (20 mmol Tris-HCl [pH 8.5], 100 mmol KCl, 10 mmol imidazole, 10 mmol 2-mercaptoethanol, 10% [vol/vol] glycerol). Recombinant proteins without the His tag were purified after direct cleavage of the His tag from the column-bound protein by treatment with approximately 1,000 U of recombinant tobacco etch virus (rTEV) protease per 3 mg of bound fusion protein. When this procedure was used, the rTEV protease was added to 5 ml of buffer A supplemented with 1 mmol dithiothreitol and 0.5 mmol EDTA and digestion was conducted for 2 h at 30°C on a shaking platform.

The recovered proteins were concentrated to 0.5 ml with Centricon 3 or Centricon 10 concentrators (Amicon, Beverly, Mass.), reconstituted to 3.0 ml with phosphate-buffered saline (PBS), and desalted either with desalting columns (Bio-Rad) or by dialysis against 0.1 mol of NaCl in PBS. Recombinant proteins had a nearly native electrophoresis and staining with Coomassie brilliant blue. Protein concentrations were determined by absorbance at 280 nm. The purified proteins were stored in liquid nitrogen.

Purification of native SpeB from strain MGAS 1719. Native 28-kDa SpeB protease was purified from the culture supernatant of strain MGAS 1719 as described previously (14). The purified protein was >95% pure, as analyzed by SDS-PAGE and staining with Coomassie brilliant blue.

Amino-terminal sequencing of recombinant proteins. A 20-μg portion of each recombinant protein with the His tag removed by digestion with rTEV protease was used to test for the expression of recombinant proteins and to assess their purity. After SDS-PAGE, the gel was run with denatured water and the proteins were transferred to a nitrocellulose membrane (Bio-Rad) with transfer buffer. The membrane was incubated for 1 h with 0.5% blocking agent (Amersham) and then for 1 h with anti-speB monoclonal antibodies (2A3-B2-C12 (21) (1:200 dilution), rabbit polyclonal antibody raised against purified C192S myoglobin (1:5,000 dilution), or human convalescent-phase sera (1:500 dilution) obtained from patients with invasive GAS infections. All serum dilutions were made with TBS containing 1% gelatin. The membranes were then washed with 0.1% Tween–TBS and water for 10 min each and incubated with secondary antibody (1:2,000 dilution) of goat antibody conjugated to horseradish peroxidase (Bio-Rad) diluted in TBS containing 1% gelatin. The membranes were washed with 0.1% Tween–TBS and water for 10 min each, and the anti-
body-antigen interaction was visualized with Bio-Rad developing reagent. The reaction was terminated with deionized water.

Rabbit polyclonal antisera. Polyclonal antisera were raised against the SpeB C192S zymogen and propeptide by immunizing rabbits with recombinant proteins purified to apparent homogeneity. The His tag was removed by digestion with tREV protease before the animals were immunized. The sera were made under contract by standard procedures used by the supplier (Bethyl Laboratories, Montgomery, Tex.).

Streptococcal protease assays. Four assays were used to test for SpeB proteolytic activity. The streptococcal protease assay used routinely during purification is based on the ability of the enzyme to cleave bovine casein embedded in an agarose gel matrix (22). A second assay exploits the capability of streptococcal protease to cleave fibronectin and has been described previously. A third assay was used to test for the ability of active wild-type cysteine protease to process recombinant C192S zymogen, active protease was incubated at 37°C for 30 min to 2 h with purified C192S zymogen at a molar ratio of 1:1 or 1:10 (22). The samples were analyzed by SDS-PAGE, and the products were visualized by silver staining. To test for the ability of active cysteine protease to degrade the recombinant SpeB propeptide, active wild-type protease was incubated at 37°C for 2 h with purified SpeB propeptide at a molar ratio of 1:1. The samples were analyzed by SDS-PAGE, and the products were visualized in parallel by staining with Coomassie brilliant blue or Western analysis with rabbit anti-propeptide.

Immuno-dot blot analysis of human patient sera. To test for human patient antibodies directed against the purified C192S zymogen, 1.0 mg of purified recombinant protein was applied to a nitrocellulose membrane. The blot was dried briefly in air, and the membrane was incubated with 0.5% blocking agent (Amersham) for 1 h. Acute- and convalescent-phase sera were diluted 1:500 and used as the primary antibody. After 1 h of incubation with primary antibody, the blots were washed with 0.1% Tween–TBS and water for 10 min each and incubated with goat anti-human secondary antibody (1:2,000 dilution of goat antibody conjugated with horseradish peroxidase diluted in TBS containing 1% gelatin). The membranes were then washed with 0.1% Tween–TBS and water three times with TBS, and incubated for 1 h at 37°C with 50 μl of serum samples diluted in TBS. After three washes with TBS, the plates were probed for 2 h at 37°C with 50 μl of horseradish peroxidase-conjugated goat anti-human immunoglobulin G (heavy and light chains) (Bio-Rad) diluted 1:2,000 in TBS. After a further three washes, the plates were incubated with 2.2'-azino-di-[3-ethylbenzthiazoline sulfonate (6)] (ABTS; Boehringer Mannheim) as the development agent for 20 min at room temperature in the dark. The reaction was terminated by adding 20 μl of 1.75% SDS solution. The absorbance was measured at 405 nm (Microplate Autoreader EL311; Bio-Tek Instruments, Inc., Winooski, Vt.), and geometric mean titers were calculated. All samples were assayed in triplicate.

RESULTS

Expression and purification of recombinant proteins. Recombinant proteins were made as His tag fusion products in E. coli BL21 induced with IPTG. The fusion products were purified by affinity chromatography with the Ni-nitrirotiacetic acid resin matrix as described in Materials and Methods. After removal of the His tag by treatment with tREV protease, the proteins were analyzed by SDS-PAGE and silver staining. As shown in Fig. 2, the recombinant C192S zymogen, C192S inactivated mature form, and propeptide were purified to apparent homogeneity. Analysis of these three proteins by amino-terminal sequencing confirmed that the recombinant molecules had the correct amino-terminal residues. The three recombinant molecules retain two extra amino acid residues (Gly-Ala) at the amino terminus that are remnants of the His tag expression system.

The purified recombinant C192S zymogen was tested by Western blotting for immunoreactivity with rabbit polyclonal antisera raised against purified mutant proteins. Rabbit polyclonal anti-C192S zymogen reacted with recombinant C192S zymogen, recombinant propeptide, and wild-type cysteine protease (9). In contrast, anti-propeptide antibody reacted with recombinant C192S zymogen and recombinant propeptide but not with the wild-type cysteine protease (9). These data showed that the purified recombinant proteins retained immunoreactivity with rabbit polyclonal antisera raised against the mutant proteins.

Lack of proteolytic activity of the C192S zymogen and C192S 28-kDa protein. In an initial analysis of SpeB mutant proteins (22), the C192S 28-kDa form appeared to retain a low level of residual proteolytic activity against fibronectin, one of the three test substrates. This result was unexpected because several lines of biochemical evidence strongly suggested that the C192 amino acid residue was essential for enzymatic activity (5, 20). Inasmuch as the level of expression of that mutant protein was low and the purification procedure available at that time was suboptimal, the possibility existed that the residual proteolytic activity was due to a contaminating E. coli protease. To further explore this issue, we tested the purified recombinant zymogen and 28-kDa forms for proteolytic activity against bovine casein and human fibronectin. The results showed that neither recombinant molecule had detectable proteolytic activity against bovine casein (9).

A more sensitive assay was then conducted to search for retention of residual protease activity. Human fibronectin was incubated overnight with C192S zymogen, C192S 28-kDa form, and wild-type mature protease (all "activated" by the addition of 2-mercaptoethanol). The resulting products were analyzed by Western blotting. The C192S zymogen and C192S 28-kDa molecules lacked detectable protease activity, whereas the wild-type mature streptococcal cysteine protease degraded the fibronectin to a series of lower-molecular-weight products (9).

Wild-type mature cysteine protease processes C192S mutant zymogen. We next tested the ability of purified wild-type cysteine protease to process C192S mutant zymogen to a 28-kDa mature form. Activated wild-type protease was incubated at a 1:1 or 1:10 molar ratio with purified recombinant C192S mutant zymogen. The products were analyzed by SDS-PAGE and silver staining. The recombinant C192S zymogen was rapidly processed to a 28-kDa protein that comigrated with wild-type mature streptococcal cysteine protease (Fig. 3A). This proteolytic product reacted with antibody made against purified recombinant C192S zymogen but not with sera raised against purified recombinant propeptide (9). These results demonstrate that the recombinant mutant zymogen retains the appropriate tertiary structure to allow efficient processing by active streptococcal protease.
Wild-type cysteine protease degrades the recombinant propeptide. The ability of wild-type cysteine protease to degrade the recombinant propeptide was then assessed. Activated cysteine protease was incubated in a 1:1 molar ratio with purified recombinant propeptide. The products were separated by SDS-PAGE and visualized by staining with Coomassie brilliant blue; they were also analyzed by Western analysis with rabbit anti-propeptide antibody. The active cysteine protease readily degraded the recombinant SpeB propeptide under these assay conditions (Fig. 3B).

Patients with invasive disease episodes seroconvert to SpeB.

We next tested the hypothesis that patients with invasive streptococcal infections seroconvert to SpeB. The immunoreactivities of purified recombinant C192S zymogen with the His tag intact or removed by digestion with tTEV protease were compared. There was no significant difference in the reactivity of the two molecules as assessed by enzyme-linked immunosorbent assay (9). These results indicated that the His-tagged molecule could be used in these assays, thereby avoiding the additional time and expense required for removal of the His tag.

Initial assessment of seroconversion was conducted with acute- and convalescent-phase sera from four randomly chosen patients with culture-proven invasive GAS disease (invasive infection or streptococcal toxic shock syndrome) (Table 1). These patients were infected with three distinct M types (M1, M3, and M6). As shown in Fig. 4, analysis by immunoblotting with purified recombinant C192S zymogen found that all four patients seroconverted to this molecule.

To more precisely determine the level of seroconversion, acute- and convalescent-phase sera from 17 patients with invasive GAS disease episodes were studied by enzyme-linked immunosorbent assay. Infections in these patients were caused by M1 (nine patients), M3 (three patients), M4 (one patient), M6 (one patient), and two M-nontypeable organisms (Table 1). The isolate cultured from one patient was not available for study. Of the 17 patients, 14 seroconverted to the C192S recombinant zymogen. The average level of seroconversion was approximately 28-fold in geometric mean titer.

DISCUSSION

Although the streptococcal cysteine protease was first characterized in 1945 (5), it has been the subject of renewed interest by several laboratories in recent years (1, 2, 4, 10, 20). Studies have shown that the molecule is initially made as a 40-kDa zymogen and, under appropriate conditions, is transformed into a 28-kDa active cysteine protease. A series of observations stimulated analysis of the virulence attributes and structure-function relationships of SpeB. Holm et al. (12) studied patients with invasive GAS episodes and made the critical finding that individuals with low acute-phase antibody levels to SpeB in serum were more likely to die or have other bad clinical outcomes than were patients with high antibody levels. Subsequently, Kapur et al. showed that immunization of mice with purified wild-type 28-kDa cysteine protease protected them against intraperitoneal injection with a GAS strain expressing a heterologous protease variant (13). More recently, Lukomski et al. (18, 19) discovered that inactivation of the SpeB protease by molecular genetic techniques profoundly decreased the ability of GAS to cause death of mice after intraperitoneal injection. In vitro and other studies have documented that the cysteine protease activates or destroys important biological mediators (20). These observations, together with the lack of a human GAS vaccine, served in part as the catalyst for the experiments reported in this paper.

Our studies showed that the His tag expression system is a convenient and efficient strategy for generating recombinant forms of SpeB. The C192S mutant zymogen retained sufficient native structural integrity to permit ready processing to the 28-kDa mature form. Moreover, both the recombinant 40-kDa mutant zymogen and the resulting 28-kDa processed molecule retained apparently normal reactivity with polyclonal and monoclonal antibodies raised against the 28-kDa native protease. In addition, the 40-kDa mutant zymogen had apparently normal reactivity with polyclonal antibodies raised against the recombinant propeptide. Taken together, the data suggest that the C192S mutation does not significantly alter the overall conformation of the recombinant molecule relative to wild-type protein.

Active 28-kDa protease accumulates in the culture supernatant during the later stages of bacterial growth in vitro (4, 5, 8). The exact mechanism whereby the 40-kDa molecule is cleaved to generate the 28-kDa proteolytically active form in vivo is unknown. In principle, processing of the 40-kDa form in vivo could occur by autocatalytic truncation, cleavage by active 28-kDa protease or a host protease, or a combination of these routes. Elliott (6) presented evidence that protease precursor...
is converted to the active enzyme autocatalytically by treatment with sulfhydryl compounds such as mercaptans, cyanide, sulfite, or sodium borohydride. This result was confirmed by electrophoretic examination of a precursor preparation before and after incubation with sodium thioglycollate (26). However, in those experiments, precursor preparations always contained a small amount of active protease (7, 26). As a consequence, it was not possible to rule out the possibility that preformed 28-kDa protease was processing the zymogen. Liu and Elliott (16) examined the proteolytic cleavage of the zymogen to the mature form in detail by use of trypsin, subtilisin, and the streptococcal protease. In those studies, all three enzymes cleaved the zymogen to yield a nearly identical mature product. These results suggested that autotruncation was not the only mechanism that could convert the zymogen to the mature form. However, because the studies had to be conducted under conditions that also stimulate autotruncation, it was not possible to assess the contribution of each mechanism to zymogen processing. Our use of the recombinant 40-kDa zymogen that lacks protease activity permitted us to unambiguously demonstrate that in vitro, active protease processes the 40-kDa form to mature 28-kDa SpeB. These data suggest that in vivo, formation of a small amount of the enzymatically active 28-kDa molecule would be sufficient to result in the rapid conversion of a pool of preformed 40-kDa zymogen to the 28-kDa protease.

The serologic response to SpeB has been previously studied by several groups (23, 24, 28). Todd (28) studied acute- and convalescent-sera obtained from 32 patients with scarlet fever, pharyngitis, or rheumatic fever and found that although 26 individuals had anti-protease antibody in their convalescent-phase serum, in most cases the level of antibody was low. Studies by Rotta (24) documenting that humans with glomerulonephritis seroconvert to protease also demonstrated that this enzyme is expressed in vivo. Similarly, Ogburn et al. (23) examined sera obtained from patients with scarlet fever and rheumatic fever but not invasive episodes. Hence, ours is the first study to examine the serologic response to SpeB by a substantial number of patients with culture-proven GAS invasive disease. The demonstration that 14 of 17 patients seroconverted to SpeB clearly documents that the molecule is

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* STSS, streptococcal toxic shock syndrome.
* Tested with recombinant Cys192Ser SpeB zymogen.
* All acute-phase sera were collected from 1 to 7 days after the patient presented with illness.
* Serologically nontypeable for M protein.
* NC, no change.

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expressed in vivo during the course of severe invasive human infections. This observation adds to several lines of evidence suggesting that SpeB is an important GAS virulence factor in some human infections. Seroconversion occurred in patients infected with a variety of distinct M types, including M1, M3, M4, M6, and two previously unidentified M types. These results are consistent with data showing that virtually all GAS strains express immunoreactive SpeB in vitro (15).

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