Streptococcal Mitogenic Exotoxin, SmeZ, Is the Most Susceptible M1T1 Streptococcal Superantigen to Degradation by the Streptococcal Cysteine Protease, SpeB*

Mohammed M. Nooh1,5,6, Ramy K. Aziz5,1, Malak Kotb1,5, Alexey Eroshkin1, Woei-Jer Chuang*, Thomas Proft††, and Rita Kansal1,5,3

From the Departments of *Molecular Sciences and ††Surgery, University of Tennessee Health Science Center, Memphis, Tennessee 38163 and the Research Center, the Veterans Affairs Medical Center, Memphis, Tennessee 38104, the Burnham Institute, La Jolla, California 92037, the **Department of Biochemistry, National Cheng Kung University College of Medicine, Tainan 701, Taiwan, and the ‡‡Faculty of Medical and Health Sciences, University of Auckland, Auckland, New Zealand

Superantigens (SAgs) play an important role in the pathogenesis of severe invasive infections caused by Group A Streptococcus (GAS). We had shown earlier that the expression of streptococcal cysteine protease SpeB results in partial loss of the immune-stimulating activity of the native secreted GAS SAgs, namely the streptococcal pyrogenic exotoxins produced by the globally disseminated M1T1 GAS strain, associated with invasive infections worldwide. In this study, we examined the susceptibility of each of the M1T1 recombinant SAgs to degradation by rSpeB. Whereas SmeZ was degraded completely within 30 min of incubation with rSpeB, SpeG, and SpeA were more resistant and SpeJ was completely unaffected by the proteolytic effects of this protease. Proteomic analyses demonstrated that the order of susceptibility of the M1T1 SAgs to SpeB proteolysis is unaltered when they are present in a mixture that reflects their native physiological status. As expected, the degradation of SmeZ abolished its immune stimulatory activity. In silico sequence disorder and structural analyses revealed that SmeZ, unlike the three other structurally related SAgs, possesses a putative SpeB cleavage site within an area of the protein likely to be exposed to the surface. The study provides evidence for the effect of subtle structural differences between highly similar SAgs on their biological activity.

Group A streptococci (GAS)4 are Gram-positive bacteria responsible for human diseases with varying severity (1, 2). The outcome of these infections is affected by a complex interplay between several surface-associated and secreted bacterial virulence factors, and host immune defenses (3, 4). Streptococcal pyrogenic exotoxins (Spes) are well known for their superantigenic activity and have been shown to be pivotal in the pathogenesis of streptococcal toxic shock syndrome (STSS) and necrotizing fasciitis (NF, the flesh-eating disease) (5, 6). SAgs are unusual bacterial toxins that play an important role in the pathogenesis of several diseases including food poisoning, autoimmunity, and toxic shock syndrome (TSS) (2, 7). A major feature of SAgs is that they bind directly to human leukocyte antigen (HLA) class II molecules and to the T-lymphocyte receptor (TCR) causing the activation of larger numbers of T-lymphocytes, almost 1000-fold more than those activated by regular antigens (2). This unconventional mode of activation causes excessive production of pro-inflammatory cytokines that can lead to disseminated intravascular coagulation, shock, and multiple organ system failure (2, 7, 8). Whereas all GAS isolates examined thus far secrete one or more SAgs, different strains can produce very different spectra of the >24 streptococcal SAgs. One of the most clinically relevant strains, the M1T1 strain, whose appearance coincided with the resurgence of severe streptococcal infections around the world in the 1980s, has the following SAg-encoding genes: speA, speF, speG, speJ, and SmeZ (9). SpeA, a horizontally acquired SAg, that has been recently introduced into this strain, has been shown to be a major player in the pathogenesis of STSS. Another streptococcal superantigen, SmeZ, was shown to play an important role in streptococcal pathogenesis (10, 11).

M1T1 isolates obtained from patients with invasive GAS disease of varying severity carry identical spe genotypes but may differ in their SAg expression phenotype (9). Whereas the disease severity could not be correlated to the a particular M1T1 SAg, there was a highly significant inverse correlation between severity of the invasive infection and the expression of the streptococcal cysteine protease, which is an important GAS virulence factor. SpeB modifies host proteins like matrix proteins thereby facilitating bacterial invasion, and it can post-translationally cleave and activate precursors for several cytokines and important components of the coagulation pathway (12, 13). However, SpeB can also modify and/or inactivate several important GAS virulence proteins, including the anti-
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Polyclonal Rabbit anti-Spe Antibodies—Specific polyclonal rabbit antibodies to SpeA were generated as previously described (16). Antibodies to SmeZ, SpeG, and SpeE were generated by an author (T. Proft).

Effect of SpeB on rSAgs—Each of the rSAgs (200 µg/ml) was incubated with the preactivated rSpeB (200 µg/ml) at 37 °C. We generated the enzymatically active form of rSpeB by incubating the 40-kDa precursor for 8 h at 37 °C in the presence of 5 mM dithiothreitol and EDTA. To monitor the kinetics of the effect of rSpeB on rSAgs, we aliquoted samples at 0.5-, 1-, 2-, 4-, 8-, and 18-h postincubation. We stopped the SpeB enzymatic activity immediately at each time point by adding the cysteine protease inhibitor E-64 (Roche Applied Science) at a final concentration of 28 µM. The samples were frozen at −20 °C until further analyses. To check the specificity of the SpeB proteolytic activity, we also included a control where its activity was inhibited from the start of incubation by adding E-64. The digestion mixtures (rSAgs + rSpeB) were separated by SDS-PAGE and transferred onto nitrocellulose paper. We analyzed the proteolytic degradation of the rSAgs following incubation with rSpeB by Western blots using specific anti-Spe antibodies.

Proteomic Analysis of the SAg Mixture—The four recombinant M1T1 SAgS (SpeA, SpeG, SpeE, and SmeZ) were mixed in equal amounts (10 µg/ml) and treated with 20 or 200 µg/ml of preactivated SpeB. The cysteine protease inhibitor, E-64 (final concentration of 28 µM) was used to stop the reaction after 8 h. Prior to isoelectric focusing (IEF), the SAg mixture, with and without active SpeB, was lyophilized to dryness, then resuspended in 125 µl of rehydration buffer composed of 7 M urea, 2 M thiourea, 4% CHAPS, 20 mM dithiothreitol, and 0.16% of 40% Bio-Rad 3–10 ampholytes (Bio-Rad). IEF was performed in a Protean IEF cell, and the second dimension SDS electrophoresis was run on a Protean Minigel Dodeca apparatus (Bio-Rad) as detailed elsewhere (15). Protein spots, visualized by silver staining, were cut from the gels, destained, and digested with trypsin. The tryptic peptides were extracted with 60% acetonitrile/5% trifluoroacetic acid, purified on micro-C18 columns (Ziptips-Millipore), and finally eluted in the matrix solution (α-cyano-4-hydroxycinnamic acid dissolved in 50% acetonitrile/0.1% trifluoroacetic acid). To identify these proteins, we analyzed their tryptic peptide masses by MALDI-TOF MS using a Voyager DE RP (ABI) in the Stout Neuroscience Laboratory (UTHSC proteomic facility) and then we used the Aldente software to match the peptide masses to proteins in the databases.

N-terminal Sequencing of the Strep SAg following Incubation with rSpeB—N-terminal protein sequencing (Edman degradation) was performed by Midwest Analytical, Inc.

Bioinformatics Analysis of SAg Sequences—We used several bioinformatics tools to analyze the amino acid sequences of the four M1T1 SAgS as well as their secondary and tertiary structures. We aligned the primary sequences using the ClustalW analysis tool (17) in VectorNTI suite (Invitrogen). We used the Consensus Disorder Prediction (CDP) tool from the Center on Proteolytic Pathways (CPP).

This tool implements five different disorder prediction methods: COILs, REM465, and HOTLOOPs (from disEMBL) and GlobPlot (18) and DISOPRED (19). In CDP, if more than half of these applied methods calculate a residue as disordered,
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Effect of SpeB on the Functional Activity of Streptococcal Sags—For assessment of the effect of SpeB on the ability of SAgs to induce lymphocyte proliferation, peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood of healthy individuals as detailed previously (21) and stimulated with each of rSAg with or without preincubation with active rSpeB as detailed above. PBMC (10^6 cells/200 μl) were stimulated with serial 2-fold dilutions of either the tested rSAg ± rSpeB or the polyclonal mitogen, phytohemagglutinin A (PHA) at 1 μg/ml conc. T-cell proliferation was assessed by measuring [3H]thymidine uptake after 72 h of culture.

Analysis of TCR Vβ Repertoire by Flow Cytometry—Each SAg has a characteristic Vβ specificity and preferentially activates T cells expressing certain Vβ elements. We used flow cytometry for quantitative analysis of preferential expansion of lymphocytes with specific TCR Vβs using the IO Test Beta Mark TCR Vβ Repertoire kit (Beckman Coulter, Miami, FL). We used a CD3-PC5-conjugate as an additional marker to enable proper gating on T-lymphocytes only. PBMC were isolated from the blood of a healthy individual by Ficoll-Hypaque gradient centrifugation, incubated at 10^7 cells/5 ml of RPMI with 10% fetal calf serum, and stimulated with optimum concentration of each SAg (without or with rSpeB) or PHA at 1 μg/ml. After 72 h, the cells were cultured for an additional 24 h in the presence of 10 units/ml recombinant human IL-2 to allow the regeneration of modulated T-cell receptors. The cells were then harvested, washed extensively with phosphate-buffered saline containing 1% bovine serum albumin, and stained with different antibodies per the manufacturer's instructions. We gated on the CD3-PC5-blastogenic cells and performed simultaneous analysis of 3 TCR Vβs per tube using a FACSCalibur flow cytometer. We analyzed the data using Cell Quest software (Becton Dickinson, Mountain View, CA). A minimum of 30,000 cell events were acquired for the analysis.

RESULTS

Proteolytic Degradation of Individual SAgs by SpeB—The clinically relevant M1T1 strain harbors the genes encoding for SAgs: SpeA, SpeF, SpeG, SpeH, SpeJ, and SmeZ. Using conventional methods of protein detection and identification, we observed that SpeF is completely degraded in the presence of SpeB (14). Because most of the remaining SAgs viz. SpeJ, SpeG, and SmeZ are secreted at levels much below the detection limits of conventional methods (14, 22), we extended these studies with the rSAgs. SpeA, SpeJ, SpeG, and SmeZ appear to be affected differently by the rSpeB. SmeZ was most susceptible to proteolytic degradation by SpeB as early as 30-min postincubation (Fig. 1A) and was virtually undetectable after 8 h even at the lowest SpeB concentration used (Fig. 2A). Compared with SmeZ, SpeJ was the most resistant to SpeB degradation even after 18 h of co-incubation with the highest used dose of SpeB. Whereas SpeJ and SmeZ exhibited extreme resistance and susceptibility to SpeB, respectively, the susceptibility of SpeG and SpeA to the proteolytic activity of SpeB varied. After 8 h of incubation, SpeG showed slight and partial degradation by 20 and 200 μg/ml SpeB, whereas partial degradation of SpeA was only noted after 18 h of incubation and only at the highest concentration of the protease (Fig. 1, A and B). In the presence of E-64, all SAgs were completely protected from SpeB degradation (Fig. 1B).

Thus far, we have determined the differential cysteine protease activity of rSpeB on each of the four rSAgs individually. In real life, however, these proteins are coexpressed in the GAS secretome. We asked if the presence of a mixture of SAgs would have a synergistic or antagonistic effect on their susceptibility to SpeB. Because these rSAgs have similar molecular weights, we resolved them by two-dimensional gel electrophoresis before and after their incubation with the activated rSpeB. The data shown in Fig. 2A and B confirmed the higher susceptibility of SmeZ to rSpeB activity and showed that this susceptibility is...
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not altered by the presence of the other three SAgs. The susceptibility of other three SAgs in the mixture showed a similar pattern as that seen with each one of them individually: SpeG showed only partial degradation while SpeA and SpeJ remained unaffected up to 8 h of incubation with rSpeB (Fig. 2, A and B).

SpeB-driven Proteolytic Degradation of SAgs Affects their Biological Activity—Following the 8-h co-incubation with SpeB, the proliferation-inducing activity of SmeZ was reduced significantly (p = 0.003) whereas that of SpeA remained unchanged (Fig. 3A). The loss of proliferation inducing activity of SmeZ following incubation with SpeB could be inhibited by the addition of specific inhibitor E-64. The proliferation-inducing activities of SpeG and SpeJ remained unaffected by SpeB (data not shown).

The proliferation results were supported by TCR \( \beta \) analyses of the human T cells stimulated with a representative SpeB-resistant SAg, SpeA, or a SpeB-sensitive SAg, SmeZ. The skewed pattern of TCR \( \beta \) repertoire in response to stimulation with SpeA was not altered following preincubation with SpeB. By contrast, the ability of SmeZ to induce the expansion of T cells bearing TCR \( \beta\delta \), TCR \( \beta\gamma \), and TCR \( \beta\beta \), was diminished when the SAg was preincubated with SpeB (Fig. 3B), with a marked reduction in the number of activated T cells bearing TCR \( \beta\beta \) elements.

Specificity of SpeB Degradation—SpeB degraded the His tag on all the SAgs used. This was first suggested by the appearance of a slightly faster migrating species in Westerns of SAgs preincubated with active SpeB, including those that resisted its proteolytic effects (Fig. 1A), and later confirmed when we probed the blots with anti-His antibodies and were unable to detect any SAg bands (data not shown), yet the proteins were readily detected at their expected size by their specific antibodies. These results prompted us to investigate the SpeB-specific cleavage sites on each of these SAgs and to explore why only SmeZ is degraded completely while other SAgs are not.

N-terminal sequencing of the protein fragments followed by amino acid alignment revealed that all four SAgs were cleaved between the glutamate (E) and glycine (G) residues just after the histidine tag (Fig. 4). These two residues do not belong to the cloned proteins but are rather encoded by the expression vector as part of the factor Xa cleavage site that was engineered between the His tag and the cloned protein to facilitate the removal of the His tag. Although SmeZ was also initially cleaved at the same site (15 min postincubation with SpeB), it was completely degraded by 30 min.

Bioinformatics Analysis Reveals a Putative SpeB Target Loop in SmeZ—The consensus target for cleavage by SpeB has been suggested to contain a hydrophobic residue (isoleucine or valine) at the P2 site and a positively charged residue (preferably lysine) at P1 (23, 24). Bioinformatics analysis of the primary
sequence and the predicted secondary structure of the SAgs used in this study did not reveal a preferential abundance of the SpeB preferred sites in SmeZ when compared with the other SAgs. Alternatively, a plausible explanation of why SmeZ is totally degraded within an hour while SpeG, SpeA, and SpeJ are spared could lie in the folds of the tertiary structures of these proteins. Accordingly, it appears that the tertiary structure of SmeZ may make some SpeB-vulnerable targets exposed whereas these may be sequestered in other SAgs. Indeed, it was suggested that large local motions proximate to the scissile bonds are required for proteolysis and that the prime determinant for limited proteolysis is this ability to unfold locally (25).

Accordingly, we used the CDP method (see “Experimental Procedures”) to predict intrinsically disordered regions in the four rSAgs. Subsequently, we looked for the SpeB extended specificity pattern [ILVFMYW]K in regions predicted to be disordered to find potential cut sites of SpeB. The only site in disordered region was found in SmeZ, boosting the experimental observation of its selective susceptibility to rSpeB. On the other hand, the homologous area in the other SAgs was either not in a disordered region (rSpeG and rSpeJ), or was in a disordered region (rSpeA) but lacking the lysine residue and having an additional proline residue that adds steric hindrance which may interfere with proteolysis (Fig. 5). Comparing the crystal structures for SpeA and SmeZ revealed that the areas are surface exposed on loops, with the difference that this loop lacks lysine in SpeA (Fig. 5).

**DISCUSSION**

SAgs play an important role in the pathogenesis of severe invasive infections caused by Group A Streptococcus (3, 7), and their expression is tightly regulated both on the transcriptional and post-translational levels (14, 26, 27). We reported earlier that SpeB plays a pivotal role in the post-translational degradation of GAS secreted protein, which results in partial loss of proliferation-inducing activity of GAS supernatants containing a mixture of several secreted SAgs (14). In the present study, we demonstrated that SAgs vary considerably in the rate and extent of their proteolytic degradation by SpeB: while some SAgs are highly susceptible, others are resistant to the degradation by SpeB up to 18 h.

SpeB is a highly conserved GAS protein that, unlike some other phage-encoded streptococcal exotoxins, e.g. SpeA and SpeC, is encoded by chromosomal DNA. SpeB is secreted through the ExPortal, a unique single microdomain of the cellular membrane specialized to contain the Sec translocons (28) as a 40-kDa precursor that can catalyze its own processing to the enzymatically active 28-kDa form, also known as streptopain because of its close resemblance to papain. The enzyme is a cysteine protease belonging to a family of endopeptidases and shows a broad substrate specificity, including oxidized insulin B chain and other synthetic substrates (29). Although it is one of the earliest identified and most extensively studied streptococcal proteins, little is known about its cleavage sites. Most of the current knowledge about these cleavage sites comes from the enzyme effects on the natural substrates i.e. pro-SpeB, immunoglobulins, C5a peptidase, streptococcal proteins M and H (12, 13, 23), or synthetic peptides (24), but no comprehensive

**FIGURE 3.** SpeB-driven proteolytic degradation of SAgs affects their biological activity. A, T-lymphocyte proliferation inducing activity of rSmeZ and rSpeA incubated without or with 200 μg/ml rSpeB (± E64) for 8 h. The proliferation inducing activity of each SAg was tested over a wide range (500 ng to 5 pg/ml of the culture media). The results shown here, however, correspond to 2.5 and 10 ng/ml of SmeZ and SpeA, respectively. The data shown are representative of three independent experiments. Each value represents mean ± S.D. of four values. Statistical significance of the differences in the proliferation-inducing responses with or without incubation with rSpeB was determined by Student t test (two-tailed). B, SpeB selectively degrades SmeZ but spares SpeA. Expansion profiles of lymphocytes with specific T-cell receptor Vβ in response to stimulation with SmeZ and SpeA with or without incubation with 200 μg/ml of rSpeB for 8 h. The final concentrations used for stimulation of SmeZ and SpeA were 5 and 10 ng/ml of the culture media, respectively.
studies have been performed to determine a consensus SpeB cleavage site. From the few studies performed, the preferred target for SpeB favors a hydrophobic residue (isoleucine or valine) at the P2 site and a positively charged residue (preferably lysine) at P1 (23, 24). The only cleavage site that we could determine by N-terminal sequencing in our studies happens to have isoleucine (I) and glutamic acid (E) at P2 and P1, respectively. However, bioinformatics analysis of SmeZ1 (and other SemZ alleles) revealed a conserved intrinsically

FIGURE 4. Sites of cleavage of the various rSAGs by SpeB: alignment of the amino acid sequences of rSpeA2, rSpeG, rSpeJ, and rSmeZ1. All of the SAGs studied were cleaved at the N-terminal end of the first G in the protein (see arrow), thereby removing all extra amino acids added onto the protein as part of the His tag included in this expression vector. Sequences are aligned by ClustalW tool available in VectorNTI suite. Amino acids likely to be in disordered areas are shown in uppercase letters. The flexible loop encompassing the GSSYKS sequence in SmeZ1 (shown in a dashed box) is expected to be the most vulnerable site for SpeB cleavage. In the same loop, note that SpeA2 has an E instead of the K found in SmeZ1 and it is possible that this substitution may have rendered SpeA resistant to SpeB. Also, the extra P found in the SpeA but not the SmeZ1 loop, may restrict the flexibility of the loop and contribute as well to its protection from SpeB proteolysis. (SpeA2 and SmeZ1 are the specific alleles of speA and SmeZ present in the M1T1 GAS.)

FIGURE 5. Ribbon diagram of the three-dimensional structures of SmeZ (left) and SpeA (right). Each shows a flexible loop that matches the region predicted by disorder analysis (see Fig. 4 and supplemental Fig. S1); however, only the SmeZ loops has a lysine moiety likely to be at the SpeB cleavage site.
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disordered region containing a motif vulnerable to SpeB
YKSG. This motif was different in SpeA (YETG) and was
preceded by a proline moiety that makes susceptibility to
protease unlikely. In both SpeI and SpeG, the homologous
motif was totally in an ordered region and expected to be
folded. This notion that folded domains are protected from
proteolysis is experimentally supported by the fact that teth-
ering a histidine tag to the recombinant SAgs and incubating
the tagged rSAgs with enzymatically active SpeB leads to a
prompt degradation of the foreign peptide linkers, generat-
ing products with identical N termini, suggesting that these
peptide linkers were exposed to the cysteine protease
because they did not fit in the SAgs folding scheme. Access-
sibility of the proteolytic cleavage sites is also known to play
an important role in the autoprocessing of pro-speB because
four of the five auto-processing cleavage sites identified are
located on accessible loops in the SpeB structure (30).

How the selective degradation of SAgs affect the disease pro-
gression remains to be explored. Before moving from the in
vitro observations to the in vivo implications, we have to
address the question of what physiological concentrations
these toxins achieve during infection. Because the concentra-
tions of these SAgs achieved during infection are practically
difficult to determine and are variable depending on the bacte-
rial isolate and the site of bacterial infection, we decided to mix
equal amounts of recombinant SAgs. At 200 μg/ml, a concen-
tration much higher than its physiological concentration (22),
SmeZ could still be degraded by SpeB, and a SpeB concen-
tration as low as 20 μg/ml was sufficient to breakdown SmeZ to
below detectable limits. However, it is not uncommon that an
enzyme may fail to act properly when the substrate level
becomes sparse. Thus, to address the functional implications of
the differential effects of SpeB on M1T1 SAgs, we compared its
effects on the functionality of the susceptible rSmeZ versus the
resistant rSpeA. Using in vitro biochemical and biological
assays, we showed that the selective degradation of SmeZ
results in a significant loss of its superantigenic activity and its
ability stimulate specific T-cells (Fig. 3, A and B), whereas all
SpeA biological functions were practically unaffected.

The question remains: what happens during actual infec-
tions? A very relevant and complex question whose answer is
controlled by at least three major factors, the coexpression of
these proteins, their regulation, and their available space. In
earlier studies, we showed that the relative expression of SpeA
and SpeB protein and RNA is drastically and temporally
reversed after animal passage (26). The differential expression
of these proteins implies the presence of tight regulatory net-
works that affects their relative levels. High levels of SpeB
expression in tissue and skin infection sites are in contrast with
its complete shutdown in blood (31). In the initial phase of
infection via skin or tissue, the bacteria need SpeB to break
through the host tissue. Once the bacteria are in the blood,
SpeB expression can be detrimental to the bacteria as this pro-
tease degrades most of the bacterial anti-phagocytic proteins as
well as the SAgs (32–35).

Selective degradation of GAS proteins by SpeB may explain
why it is advantageous for the bacteria to down-regulate SpeB
after the initial stages of infection, but it also explains what has
been thought as “functional redundancy” of SAgic toxins in
GAS. From an evolutionary aspect, it appears that GAS con-
tinue to acquire via horizontal gene transfer, more SAgs that
seem to resist SpeB activity. The present study suggests that
SpeA and SpeI might be two such horizontally acquired SAgs
because of 2 reasons: 1) their location on phage or transposable
elements and 2) their absence in many sequenced GAS strains.
Both SpeA and SpeI are not shared by all strains (SpeI is present
in M1 (36) and M28 (37), and SpeA in only 20% of GAS strains
(38)). These superantigens also turn out to be most resistant to
SpeB degradation and are able to keep their biological functions
regardless whether SpeB is expressed or not. It is believed that
nonspecific immune responses elicited by the SAgs may allow a
subset of the bacteria to escape specific immune defenses and
make their way to host niches where they can cause chronic
diseases such as rheumatic heart disease, glomerulonephritis,
psoriasis, and pediatric neurological disorders (39).

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