\( \alpha_2 \)-Macroglobulin-Proteinase Complexes Protect \textit{Streptococcus pyogenes} from Killing by the Antimicrobial Peptide LL-37

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The significant human bacterial pathogen \textit{Streptococcus pyogenes} expresses GRAB, a surface protein that binds \( \alpha_2 \)-macroglobulin (\( \alpha_2 \)M), a major proteinase inhibitor of human plasma. \( \alpha_2 \)M inhibits proteinase by trapping the proteinase, which, however, still remains proteolytically active against smaller peptides that can penetrate the \( \alpha_2 \)M-proteinase complex. Here we report that SpeB, a cysteine proteinase secreted by \textit{S. pyogenes}, is trapped by \( \alpha_2 \)M bound to protein GRAB. As a consequence, SpeB is retained at the bacterial surface and protects \textit{S. pyogenes} against killing by the antibacterial peptide LL-37.

\textit{Streptococcus pyogenes} is a common and clinically important human bacterial pathogen causing a wide range of invasive and non-invasive disease, as well as non-suppurative sequelae (for review, see Ref. 1). Work in many laboratories has underlined the significance of proteolysis in \textit{S. pyogenes} virulence (for review, see Ref. 2). These Gram-positive bacteria secrete proteinases and they inhibit/activate proteolytic cascades of the human host, including the complement, contact, coagulation, and fibrinolytic systems. As \textit{S. pyogenes} penetrates into normally sterile sites, this will attract neutrophils, which, when activated, will release proteinases. Proteinases also leak from damaged cells and tissues into the infectious nidus. Combined this generates massive proteolytic activity, which has been suggested to promote \textit{S. pyogenes} virulence by enhancing tissue spread and dissemination. However, to exploit this activity efficiently, the bacteria should also be able to regulate and modify the proteolysis they induce. It was therefore interesting to find that \textit{S. pyogenes} expresses a surface protein called GRAB, which binds the proteinase inhibitor \( \alpha_2 \)-macroglobulin (\( \alpha_2 \)M) with high specificity and affinity (3). \( \alpha_2 \)M bound to the bacterial surface via GRAB entraps and inhibits host and \textit{S. pyogenes} proteinases and thereby protects bacterial surface proteins and virulence determinants from degradation (3). Among these proteinases is SpeB, a classical enzyme of \textit{S. pyogenes} (4).

SpeB is a cysteine proteinase that is secreted in large quantities by \textit{S. pyogenes}. It has broad proteolytic activity and cleaves a large number of human proteins as well as surface proteins of \textit{S. pyogenes} (for review, see 2). Like several other secreted bacterial proteinases, SpeB degrades and inactivates LL-37 (5), one of the major human antibacterial peptides (for review, see Ref. 6). LL-37 is small enough (4.5 kDa) to penetrate into the cage that \( \alpha_2 \)M forms around the trapped proteinase, which commonly remains proteolytically active in the cage. The hypothesis that active SpeB could be retained at the bacterial surface in complex with \( \alpha_2 \)M and protect \textit{S. pyogenes} against killing by LL-37 initiated the present investigation.

EXPERIMENTAL PROCEDURES

Proteins and Peptides—The streptococcal cysteine proteinase was purified as described previously (7). The activity of the purified protein was examined by active site titration as described (8). The SpeB preparation used in this paper was 46% active. Throughout the paper the amount of active SpeB is given. To activate SpeB, the proteinase was incubated in activation buffer (1 mM EDTA, and 1 mM DTI in 0.1 M NaAc-HAc, pH 5.0) for 30 min at 37 °C, and activated SpeB was used throughout this investigation. Since high concentrations of DTT were found to reduce the inhibitory capacity of \( \alpha_2 \)M, the amount of DTT used in the assays was reduced compared with previous investigations (7, 8). The final concentration of DTT in all assays was 0.1 mM, which did not affect SpeB activity toward azocasein. To block SpeB activity, 500 units (Sigma) was added to the protease to a final concentration of 10 μM. 

SpeB was purified as described previously (7). The activity of the purified protein was determined using the Image Gauche program. For competition experiments, radiolabeled SpeB and different amounts of proteinase were added to \( \alpha_2 \)M simultaneously. The reactions were terminated by the addition of SDS-PAGE sample buffer and the samples were put on ice.

Analysis of Proteolysis—SpeB was mixed with 2.5 μg of \( \alpha_2 \)M or 1 μl of heparinized human plasma in 10 μl of PBS and followed by incubation for 15 min at 37 °C. The mixture was subjected to SDS-PAGE using non-reducing conditions, followed by autoradiography using a BAS-III imaging plate. The plate was scanned with a Bio-Imaging Analyzer BAS-2000 (Fuji Photo Films Co. Ltd.), and intensity was calculated using the Image Gauche program. For competition experiments, radiolabeled SpeB and different amounts of proteinase were added to \( \alpha_2 \)M simultaneously. The reactions were terminated by the addition of SDS-PAGE sample buffer and the samples were put on ice.

The abbreviations used are: \( \alpha_2 \)M, \( \alpha_2 \)-macroglobulin; DTT, dithiothreitol; PBS, phosphate-buffered saline; Z, benzylxoycarbonyl; AFC, 7-amino-4-trifluoromethylcoumarin; Tricine, N-(2-hydroxy-1,1-bis(hydroxymethyl)ethyl)glycine; cfu, colony-forming unit.
spectrometer (PerkinElmer Life Sciences). SpeB activity against LL-37 was determined by incubating 1.25 pmol of Texas Red-labeled LL-37 with 10 pmol of SpeB preincubated with PBS or 25 pmol of α₂M. After various time points aliquots were taken, and E64 was added to inactivate SpeB. The samples were separated by SDS-PAGE on a 16.5% Tris-Tricine gel (reducing conditions).

**SpeB Binding Assays—** S. pyogenes of strain KTL3 or its derivative MR4 (lacking GRAB at the bacterial surface) were grown to mid-log phase (A₆₀₀ = 0.5), washed in PBS, and resuspended in PBS to a final concentration of 5 × 10⁸ cfu/ml. The bacteria were preincubated for 30 min with PBS or α₂M, washed three times in PBS, and resuspended in PBS. Radiolabeled SpeB was activated and preincubated for 15 min with PBS or E64 and added to the bacterial suspensions. After 15 min, the bacteria were washed three times, and the radioactivity of bacterial pellets was determined. Alternatively, incubation proceeded for various time points after the washing steps, and the radioactivity in the pellets was determined as described above.

**LL-37 Killing Assays—** KTL3 or MR4 bacteria were grown to mid-log phase (A₆₀₀ = 0.5), washed, and resuspended in 0.1 M Tris, pH 7.5, with 10 mM glucose (Tris-glucose). LL-37 (100 pmol) was pretreated for 1 h with different concentrations of SpeB, or SpeB complexed with α₂M, and added to the bacterial suspension. After 2 h, the bacteria were washed in Tris-glucose, and viable counts were performed. Alternatively, for determining the protective effects of the α₂M-SpeB complex bound to the bacterial surface via GRAB, KTL3 or MR4 bacteria were pretreated with α₂M for 30 min. The bacteria were washed three times in Tris-glucose and incubated with activated SpeB for 15 min. After three additional washes, different concentrations of LL-37 were added, and viable counts were performed after 2 h.

**RESULTS AND DISCUSSION**

Initial experiments were performed to expand previous studies (3) on the relationship between SpeB and α₂M. Proteinase entrapment by α₂M results in inhibition of proteinase activity against larger protein substrates, and when SpeB was incubated with different concentrations of α₂M, a dose-dependent inhibition of SpeB activity against azocasein was observed (Fig. 1A). The SpeB-α₂M interaction was further investigated by mixing radiolabeled SpeB with α₂M or plasma, followed by SDS-PAGE and autoradiography. A fraction of the radiolabeled SpeB co-migrated with α₂M, demonstrating the formation of α₂M-proteinase complexes (Fig. 1B). Pretreatment of α₂M or plasma with methylamine, converting α₂M to an inactive state, or pretreatment of radiolabeled SpeB with the cysteine proteinase inhibitor E64, prevented complex formation, as did the addition of a molar excess of unlabeled SpeB (Fig. 1B).

Proteinases are captured by α₂M after cleaving a bait region within the α₂M molecule. To determine the cleavage site of SpeB in α₂M, SpeB was incubated with α₂M and the resulting α₂M-SpeB complexes were subjected to SDS-PAGE. The cleavage pattern indicated that SpeB cleaved α₂M in the bait region (data not shown), which was confirmed by NH₂-terminal sequencing of excised protein bands. A sequence corresponding to the α₂M bait region was obtained, identifying a cleavage site between His⁶⁹⁹ and Val⁷⁰⁰. Papain has the same cleavage site (12), further emphasizing the similarity in substrate specificity between SpeB and papain (13, 14).

The SpeB trap assay was used to investigate the time-dependence of the interaction between radiolabeled SpeB and a molar excess of α₂M. The association between SpeB and α₂M followed a hyperbolic curve reaching 50% association after ~1 min (Fig. 1C). Both trypsin and plasmin are efficiently inhibited by α₂M, and unlabeled trypsin, plasmin, and SpeB were used to compete with the binding of radiolabeled SpeB to α₂M (Fig. 1D). SpeB and trypsin had similar competition profiles, whereas plasmin was less efficient in inhibiting α₂M-SpeB complex formation under the conditions used.

These data show that SpeB is inactivated by α₂M when tested against azocasein in solution, and previous work has demonstrated that SpeB cleaves and inactivates the antimicrobial peptide LL-37 (5). Since proteinases in complex with α₂M generally retain their activity against small substrates, we investigated whether SpeB in complex with α₂M was still active against LL-37. SpeB was pretreated with a molar excess of α₂M, followed by incubation with Texas Red-labeled LL-37. Samples were taken at various time points and separated by SDS-PAGE (Fig. 2A). The degradation pattern of LL-37 by SpeB is very similar to that of Pseudomonas aeruginosa elastase (5), which cleaves LL-37 in the region responsible for its antibacterial activity (15). Unexpectedly, LL-37 was cleaved more efficiently when incubated with SpeB-α₂M complexes than with only SpeB. α₂M alone did not induce degradation of LL-37 (data not shown). When the activity of SpeB against the small fluorescent substrate Z-Arg-AFC (11) was tested, SpeB in complex with α₂M was found to be three to five times more

**Fig. 1. SpeB is trapped by α₂M.** A, α₂M inhibits SpeB activity against azocasein. SpeB (12 pmol) was incubated with different concentrations of α₂M for 15 min, and the enzymatic activity against azocasein was measured. Values from a representative experiment are depicted. B, α₂M-SpeB association is dependent on α₂M configuration and SpeB activity. Radiolabeled SpeB was mixed with purified α₂M (left gel) or plasma (right gel) for 15 min, separated by SDS-PAGE under non-reducing conditions, and subjected to autoradiography. The wells were loaded as follows: radiolabeled SpeB only (a), radiolabeled SpeB and α₂M/plasma (b), radiolabeled SpeB and α₂M/plasma pretreated with methylamine (c), radiolabeled SpeB and α₂M/plasma treated with an excess of unlabeled SpeB (d), and radiolabeled SpeB pretreated with E64 and α₂M/plasma (e). The high molecular weight bands correspond to SpeB complexes with α₂M and the low molecular weight bands to uncomplexed SpeB. C, time course of the SpeB-α₂M complex formation. Radiolabeled SpeB was mixed with an excess of α₂M and allowed to react for different periods of time, followed by SDS-PAGE (reducing conditions) and autoradiography. Values are given as percent of maximum association of SpeB to α₂M (n = 3 ± S.D.). D, proteinase competition experiments. Different amounts of unlabeled SpeB (a), trypsin (b), or plasmin (c) were added together with radiolabeled SpeB to 0.7 pmol of α₂M and allowed to react for 15 min, followed by SDS-PAGE (reducing conditions) and autoradiography. Values from a representative experiment are depicted and given as percent of association of SpeB to α₂M in the absence of competitor.
active than SpeB alone (data not shown). The molecular basis for the enhanced peptide degradation by SpeB in complex with α₂M is unclear, but the observation was further substantiated in experiments with *S. pyogenes* bacteria. Thus, α₂M-SpeB complexes inhibited LL-37-mediated killing more efficiently than SpeB alone (Fig. 2B). Again, α₂M alone had no effect (Fig. 2B, 0 pmol of SpeB, filled bar).

The results described above show that SpeB in complex with α₂M effectively degrades LL-37 and that the presence of the complexes reduces the ability of LL-37 to kill *S. pyogenes* bacteria. *S. pyogenes* induces potent inflammatory responses leading to increased vascular permeability and leakage of plasma proteins, including α₂M, into the site of infection. As mentioned, SpeB has broad substrate specificity, and when secreted the enzyme will encounter a complex mixture of proteins. It seems unlikely that a non-selective cleavage of a large number of potential substrates would be advantageous for the pathogen. By forming complexes with α₂M a much more restricted activity, focused on small peptides like LL-37, is achieved. However, to be fully effective against antibacterial peptides attacking the bacterial cell membrane, the proteolytic activity should probably also be located at the bacterial surface.

Most strains of *S. pyogenes* express GRAB, an α₂M-binding cell wall-attached protein (3, 16). To determine whether GRAB could mediate binding of SpeB to the bacterial surface via α₂M, radiolabeled SpeB was added to the *S. pyogenes* strain KTL3 or to MR4, a mutant of KTL3 lacking GRAB (3), that had been pretreated with PBS (a), KTL3 bacteria were pretreated with α₂M (b), KTL3 bacteria were pretreated with α₂M and then mixed with radiolabeled SpeB inactivated with E64 (c), and MR4 bacteria were pretreated with α₂M (d). Values are the means of three experiments ± S.D. B, time-dependent binding of SpeB to the surface of *S. pyogenes* bacteria. KTL3 (■) or MR4 (○) bacteria were incubated with α₂M and washed, and radiolabeled SpeB was added. After 15 min, the bacteria were washed and resuspended in PBS. Aliquots taken at different time points were pelleted by centrifugation, and the radioactivity of the pellets was determined. Data are from a representative experiment. C, effect of surface bound α₂M-SpeB complexes on LL-37-mediated killing. KTL3 (■) or MR4 (○) bacteria were incubated with α₂M and washed. SpeB was added followed by incubation (15 min) and washing. Different concentrations of LL-37 were added. The incubation proceeded for 2 h, and the number of cfus was determined. The values are the means from three experiments ± S.D.

To investigate whether α₂M-SpeB complexes at the bacterial surface protect *S. pyogenes* against killing by LL-37, mid-log cultures of KTL3 or MR4 bacteria were incubated with α₂M, followed by SpeB, and then washed. Subsequently, different amounts of LL-37 were added to the bacteria. After 2 h, the number of colony-forming units was determined (Fig. 3C). Compared with MR4 bacteria, KTL3 bacteria preincubated with α₂M survived significantly higher concentrations of LL-37. No difference in sensitivity to LL-37 between the bacterial strains was seen without preincubation with α₂M and SpeB (data not shown).

Fig. 4 summarizes the observations of this study, demonstrating that α₂M-SpeB complexes can be formed at the surface...
of *S. pyogenes* through protein GRAB and that these complexes protect the bacteria from killing by LL-37. Previously, other mechanisms for *S. pyogenes* defense against antimicrobial peptides have been described. SIC, another secreted protein of *S. pyogenes* (17), inactivates both LL-37 and the neutrophil-derived antimicrobial peptide α-defensin (18). Moreover, apart from cleaving LL-37, SpeB degrades proteoglycans, thereby releasing dermatan sulfate that binds to and inactivates α-defensin (19). Thus, *S. pyogenes* has developed different defense strategies against antimicrobial peptides. This apparent redundancy could be explained by the temporal aspects of an *S. pyogenes* infection. It has been proposed that the early phase of *S. pyogenes* infection is characterized by inhibition of proteolytic activity, including inhibition of complement activation, at the bacterial surface, while later phases are characterized by massive proteolysis due to the release of SpeB and host proteinases (2). In this context, SIC would be an important early defense against antimicrobial peptides, being produced in the early growth phase (17). As the infection proceeds, SpeB production will start and soluble and surface bound α,M-SpeB complexes are formed. Thus, at this stage bacterial surface proteins are protected, and SpeB activity is directed against smaller substrates, like antimicrobial peptides. When SpeB production further increases, the protective mechanism is overridden and bacterial surface proteins responsible for bacterial attachment to host structures will be degraded, probably facilitating bacterial spread (2,8). When the local α,M pool is depleted, SpeB will degrade proteoglycans, thus generating free dermatan sulfate that inactivates α-defensin. At this late phase of infection, activation of potent host proinflammatory systems by SpeB (20,21) will induce clinical symptoms of *S. pyogenes* disease. Since the discovery of antibacterial peptides (22), the significance of these peptides in the initial clearance of bacteria at epithelial surfaces has been firmly established (for review, see Refs. 23–26). It has also become increasingly evident that proteolysis and inhibition of proteolysis contribute to bacterial pathogenicity and virulence. The finding that α,M-SpeB complexes protect *S. pyogenes* from killing by LL-37 represents a novel principle for the evasion of innate immunity and unites these lines of research. In this context, it is interesting that α,M-like proteins were recently identified in a large number of bacterial species (27), further emphasizing the need for regulation of proteolysis also in bacteria. Notably, an α,M homologue was not found in *S. pyogenes* (27), which, on the other hand, can bind α,M via protein GRAB. These observations support the notion that entrapment of proteinases through α,M and α,M-like proteins could be a widespread mechanism to direct bacterial proteolysis against smaller substrates, such as antibacterial peptides.

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**REFERENCES**

1. Cunningham, M. W. (2000) Clin. Microbiol. Rev. 13, 470–511.
2. Rasmussen, M., and Björck, L. (2002) Mol. Microbiol. 43, 537–544.
3. Rasmussen, M., Müller, H. P., and Björck, L. (1999) J. Biol. Chem. 274, 15338–15344.
4. Elliott, S. D. (1945) J. Exp. Med. 81, 573–592.
5. Schmidtchen, A., Frick, I. M., Andersson, E., Tapper, H., and Björck, L. (2002) Mol. Microbiol. 46, 157–168.
6. Annetti, M. (2004) J. Leukocyte Biol. 75, 39–48.
7. Bergé, A., and Björck, L. (1995) J. Biol. Chem. 270, 9662–9667.
8. Nyberg, P., Rasmussen, M., Von Pawel-Rammingen, U., and Björck, L. (2004) Microbiology 150, 1559–1569.
9. Muller, H. P., and Rantamaki, L. K. (1995) Infect. Immun. 63, 2833–2839.
10. Barrett, A. J., Brown, M. A., and Sayers, C. A. (1979) Biochem. J. 181, 401–418.
11. Musser, J. M., Stockhauer, K., Kapur, V., and Rudgers, G. W. (1996) Infect. Immun. 64, 1913–1917.
12. Sattrup-Jensen, L., Sand, O., Kristensen, L., and Fey, G. H. (1989) J. Biol. Chem. 264, 15781–15789.
13. Collin, M., and Olsen, A. (2001) Infect. Immun. 69, 7187–7189.
14. Nomizu, M., Pietrzyński, G., Kato, T., Lachance, P., Menard, R., and Ziemek, E. (2001) J. Biol. Chem. 276, 44551–44556.
15. Oren, Z., Lerman, J. C., Gudmundsdóttir, G. H., Agerberth, B., and Shai, Y. (1999) Biochim. Biophys. Acta 1411, 501–513.
16. Toppel, A. W., Rasmussen, M., Rohe, M., Medina, E., and Chhatwal, G. S. (2005) J. Infect. Dis. 187, 1894–1895.
17. Akeson, P., Sjöholm, A. G., and Björck, L. (1996) J. Biol. Chem. 271, 1081–1088.
18. Frick, I. M., Akeson, P., Rasmussen, M., Schmidtchen, A., and Björck, L. (2003) J. Biol. Chem. 278, 16561–16566.
19. Schmidtchen, A., Frick, I. M., and Björck, L. (2003) Mol. Microbiol. 39, 708–713.
20. Kapur, V., Majesky, M. W., Li, L. L., Black, R. A., and Musser, J. M. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 7676–7680.
21. Herwald, H., Collin, M., Müller-Esterl, W., and Björck, L. (1996) J. Exp. Med. 184, 665–673.
22. Steiner, H., Hultmark, D., Engström, A., Bennich, H., and Roman, H. G. (1981) Nature 292, 246–248.
23. Selsted, M. E., and Ouellette, A. J. (1995) Trends Cell Biol. 5, 114–119.
24. Lehrer, R. I., and Ganz, T. (1999) Curr. Opin. Immunol. 11, 25–37.
25. Schröder, J. M., and Harder, J. (1999) Int. J. Biochem. Cell Biol. 31, 645–651.
26. Román, H. G. (2000) Immunol. Rev. 173, 5–16.
27. Budd, A., Blandin, S., Levashina, E. A., and Gibson, T. J. (2004) Genome Biology http://genomebiology.com/2004/05/15/RS.