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Streptococcal Cysteine Proteinase Releases Kinins: a Novel Virulence Mechanism

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Summary

Previous work has indicated a crucial role for the extracellular cysteine proteinase of *Streptococcus pyogenes* in the pathogenicity and virulence of this important human pathogen. Here we find that the purified streptococcal cysteine proteinase releases biologically active kinins from their purified precursor protein, H-kininogen, in vitro, and from kininogens present in the human plasma, ex vivo. Kinin liberation in the plasma is due to the direct action of the streptococcal proteinase on the kininogens, and does not involve the previous activation of plasma prekallikrein, the physiological plasma kininogenase. Judged from the amount of released plasma kinins the bacterial proteinase is highly efficient in its action. This is also the case in vivo. Injection of the purified cysteine proteinase into the peritoneal cavity of mice resulted in a progressive cleavage of plasma kininogens and the concomitant release of kinins over a period of 5 h. No kininogen degradation was seen in mice when the cysteine proteinase was inactivated by the specific inhibitor, Z-Leu-Val-Gly-CHN_2, before administration. Intraperitoneal administration into mice of living *S. pyogenes* bacteria producing the cysteine proteinase induced a rapid breakdown of endogenous plasma kininogens and release of kinins. Kinins are hypotensive, they increase vascular permeability, contract smooth muscle, and induce fever and pain. The release of kinins by the cysteine proteinase of *S. pyogenes* could therefore represent an important and previously unknown virulence mechanism in *S. pyogenes* infections.

Streptococcus pyogenes causes suppurative infections such as acute pharyngitis, impetigo, and erysipelas whereas glomerulonephritis and rheumatic fever are clinically important sequelae following these acute infections. Since the late 1980's an increase of toxic and severe *S. pyogenes* infections has been reported worldwide (1), and observations in various laboratories have suggested that an extracellular cysteine proteinase produced by *S. pyogenes* may contribute to this hyperacute and often lethal toxic shock-like syndrome.

The streptococcal cysteine proteinase (SCP)[1] was the first prokaryotic cysteine proteinase to be isolated and this early work also demonstrated that the enzyme has profibrinolytic activity (2). More recently, Gerlach et al. (3) found that SCP is identical to erythrogenic toxin B, one of the classical toxins of *S. pyogenes*. Experimental infections in mice indicated that SCP is an important virulence determinant (4, 5) and patients with fatal *S. pyogenes* infections have lower antibody titers to SCP in the acute phase than patients with less severe infections (6). Moreover, the enzyme activates human interleukin-1β (7), a major cytokine mediating inflammation and shock. SCP also degrades human extracellular matrix proteins (8) and releases biologically active fragments of surface proteins expressed by *S. pyogenes* (9). One of these fragments, derived from the streptococcal C5a peptidase (10), blocks the recruitment of leukocytes to the site of infection (9). SCP is also thought to inhibit cell migration by the proteolytic cleavage of the urokinase receptor exposed on the surface of mononuclear phagocytes (11). An extracellular product of *S. pyogenes* referred to as nephritis-associated protein, is identical to the inactive zymogen form of SCP (12) that is rapidly activated upon injection into the mouse peritoneum (Cooney, Liu, and Björck, in preparation). Combined, these various experimental data suggest an important role for SCP in virulence.

[1]*Abbreviations used in this paper:* D, kininogen domain; DTT, dithiothreitol; E-64, L-*trans*-epoxysuccinyl-leucylamido(4-guanidino)butane; fura-2/AM, 1-[2-(5-carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxy]-2-(2′-amino-5′-methylphenoxy)-ethane-*N,N,N′,N′*-tetra acetic acid, pentaacetoxymethylester; SCP, streptococcal cysteine proteinase.

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Kinins are potent pro-inflammatory peptides that mediate vasodilatation, spasm, pain, fever, and edema due to increased vascular permeability (13). Under physiological conditions, the kinins are released from their large multifunctional precursor proteins, high molecular weight (H-)kininogen and low-molecular-weight (L-)kininogen, by the proteolytic action of the kallikreins (14, 15), see Fig. 1. In a recent study, a majority of *S. pyogenes* strains was found to bind kininogens with high affinity and specificity (16) and a goal of the present work was therefore to investigate whether SCP can liberate kinins from the kininogens in vitro and in vivo. The release of highly potent pro-inflammatory host peptides such as kinins may explain in part the hyperacute and severe symptoms of the toxic shock syndrome. Our results demonstrate that SCP indeed has this capacity.

Materials and Methods

Bacterial Strains. *S. pyogenes* strains AP1 (40/58) and AP74 (30/50) are from the World Health Organisation Collaborating Centre for References and Research on *Streptococci*, Institute of Hygiene and Epidemiology (Prague, Czech Republic).

Sources of Proteins and Antibodies. H-kininogen was isolated from human plasma (17) with modifications previously described (18). The streptococcal cysteine proteinase (SCP) was purified from the culture medium of strain AP1 (9). The AP1 supernatant was subjected to ammonium sulfate precipitation (80%) followed by fractionation on S-Sepharose in a buffer gradient (5–250 mM MES, pH 6.0). The zymogen was further purified by gel filtration on Sephadex G-200 (9). Monoclonal antibodies to human kininogens (HKH 15 and HKL 9) were produced in mice (19), polyclonal antiserum (AS88) to human H-kininogen in sheep (20), and polyclonal antiserum against the streptococcal cysteine proteinase were raised in rabbits. Antiserum to bradykinin (α-BK, AS348) was produced in a rabbit by previous coupling of the cognate peptides to keyhole limpet hemocyanin (KLH) via the carbodiimide method (21). Peroxidase-conjugated goat anti–rabbit, goat anti–mouse (Bio-Rad, Richmond, CA), or donkey anti–sheep immunoglobulins (ICN, Aurora, OH) were used as secondary antibodies. The Z-Leu-Val-Gly-CHN_2 peptide has been described (4).

Cleavage of H-kininogen by SCP. H-kininogen (0.5 mg/ml) was incubated at 37°C with SCP in 10 mM NaH_2PO_4, 10 mM Na_2HPO_4, 0.15 M NaCl, pH 7.4 (PBS) containing 1 mM dithiothreitol (DTT); the molar ratio of substrate over enzyme was 100:1 or 1:1. Aliquots (8 μl) of the reaction mixture were removed at the indicated time points, and the reaction stopped by adding 10 μl of a 2% (wt/vol) sodium dodecyl sulfate (SDS) sample buffer (22) containing 5% (vol/vol) 2-mercaptoethanol, and boiling at 95°C. Alternatively the reaction was stopped by addition of 10 μM (final concentration) of *N*-[*N*-(L-3-transcarboxyoxiran-2-carbonyl)-L-leucyl]-agmatin (E-64).

Cleavage of Plasma Prekallikrein by SCP. Plasma prekallikrein (16 μg) was incubated with 0.05–0.5 μg of SCP in 100 μl of PBS containing 1 mM DTT at 37°C for 60 min; the molar ratio was 10:1 to 100:1. The reaction was stopped by adding 10 μl of SDS sample buffer containing 5% 2-mercaptoethanol and boiling at 95°C; alternatively E-64 was added to a final concentration of 10 μM.

Prekallikrein Activation. Plasma prekallikrein (4 μg) was incubated for 1 h with 0.012 μg factor XIIa in 40 μl of PBS, or for 3 h with varying amounts (0.12–0.012 μg) of SCP at 37°C. To test the activity of the generated proteinase, kallikrein was added to 200 μl of a 0.6 mM solution of S-2302 (H-D-Pro-Phe-Arg-*p*-nitro-anilide; Haemochrom Diagnostica, Essen, Germany) in 0.15 M Tris-HCl, pH 8.3. The substrate hydrolysis was measured at 405 nm.

Cleavage of Plasma Proteins by SCP. 100 μl of human plasma was incubated with 3.2 μg of SCP dissolved in 100 μl PBS, 10 mM DTT, pH 7.4, at 37°C. The reaction was stopped by the addition of 100 μl of SDS sample buffer containing 5% 2-mercaptoethanol (22) and boiling at 95°C for 5 min.

SDS–polyacrylamide Gel Electrophoresis (PAGE). Proteins were separated by 10 or 12.5% (wt/vol) polyacrylamide gel electrophoresis in the presence of 1% (wt/vol) SDS (22). Standard molecular weight markers were from Sigma Chem. Co (St. Louis, MO).

Western Blotting and Immunoprinting. Proteins were resolved by SDS-PAGE and transferred onto nitrocellulose membranes for 30 min at 100 mA (23). The membranes were blocked with 50 mM KH_2PO_4, 0.2 M NaCl, pH 7.4, containing 5% (wt/vol) dry milk powder and 0.05% (wt/vol) Tween 20. Immunoprinting of the transferred proteins was done according to Towbin et al. (24). The first antibody was diluted 1:1000 in the blocking buffer (see above). Bound antibody was detected by a peroxidase-conjugated secondary antibody against sheep, rabbit or mouse immunoglobulin followed by the chemiluminescence detection method.

Ca^{2+} Release from Intracellular Stores. Human foreskin fibroblasts (HF-15) on 10-mm diameter glass coverslips were grown to confluency in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal calf serum (25). The cells were washed twice with minimum essential medium buffered with 20 mM Na^+-Hepes, pH 7.4 (buffer A; without vitamins, and α-D-glucose added immediately before use). The cells were loaded for 30 min at 37°C with 2 μM 1-[2-(5-carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxy]-2-(2′-amino-5′-methylphenoxy)-ethane-*N,N,N′,N′*-tetra acetic acid, pentaacetoxymethylester (fura-2/AM; Calbiochem Novabiochem, San Diego, CA) in buffer A containing 0.04% (wt/vol) of the nonionic detergent pluronic F-127 (Calbiochem Novabiochem) (25). The cells were washed twice with buffer A. The Hitachi F4500 fluorescence photometer was employed with the

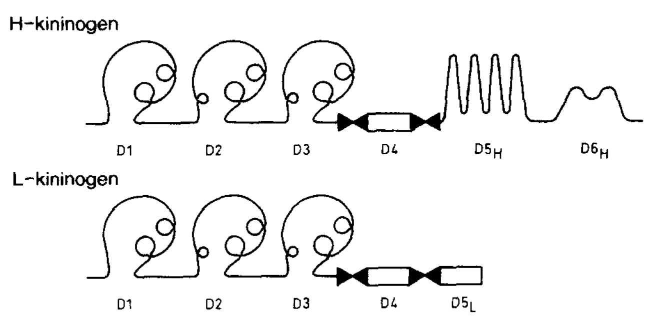

Figure 1. Gross structure of mammalian kininogens. H-kininogen and L-kininogen share their heavy chain domains, D1 to D4, and differ in their light chain domains, $D5_H$/$D6_H$ and $D5_L$, respectively. Domains D1 to D3 are of cystatin-like structure; domain D2 inhibits calpain and papain-like cysteine proteinases whereas D3 inhibits only papain-like enzymes and exposes a cell binding site. The kinin segment is located in domain D4. Domain $D5_H$ of H-kininogen exposes a high-affinity cell binding site which is also used by streptococcal M protein. Domain $D6_H$ contains the overlapping binding sites for prekallikrein and factor XI. The function of $D5_L$ of L-kininogen is unknown. Proteinase-sensitive regions flanking the kinin segment are indicated by pairs of solid arrowheads.

excitation wavelength alternating between 340 nm and 380 nm, and the emission wavelength set at 510 nm. To induce the Ca^{2+} release, 2 μg H-kininogen or proteolytic cleavage products thereof in 20 μl of reaction buffer was added 60 s after starting the measurement. The release of Ca^{2+} from intracellular stores was followed for 300 s; the free intercellular Ca^{2+} concentration was calculated from the ratio of 340 nm/380 nm as described (25).

Determination of Kinin Concentrations in Plasma. To measure the SCP-induced kinin release 100 μl of plasma was incubated with 3.2 μg of SCP in 100 μl PBS containing 10 mM DTT. Samples (10 μl each) were removed after 0, 30, 60, 90, and 120 min. The reaction was stopped by adding E-64 to a final concentration of 10 μM. For control 100 μl of human plasma was incubated with buffer in the absence of SCP. The samples were diluted 1:100 in distilled water. Aliquots (100 μl each) were mixed with 20 μl of 20% (wt/vol) trichloroacetic acid and centrifuged at 1,500 *g* for 10 min. The kinin concentrations in the reaction mixtures were quantitated by the Markit-A kit (Dainippon Pharmaceutical Co., Osaka, Japan) as described (26). Briefly, aliquots of the supernatant (75 μl each) were mixed with 75 μl of the kit buffer, and applied to the wells (100 μl each) of microtiter plates that were coated with capture antibodies to rabbit immunoglobulin followed by specific anti-bradykinin antibodies. After 1 h of incubation, the peroxidase-labeled bradykinin probe was applied and incubated for 1 h. The amount of bound peroxidase was visualized by the substrate solution, 0.1% (wt/vol) diammonium-2,2′-azinobis-(3-ethyl-2,3-dihydrobenzthiazoline)-6-sulfonate (ABTS), 0.012% (vol/vol) H_2O_2 in 100 mM citric acid, 100 mM NaH_2PO_4, pH 4.5, for 30 min. The change of absorbance was read at 405 nm. The reference standards were prepared according to the manufacturer's instructions.

Animal Experiments. *S. pyogenes* of strains AP1 and AP74 were grown in Todd-Hewitt broth (Difco, Detroit, MI) at 37°C for 16 h, and harvested by centrifugation at 3,000 *g* for 20 min. The bacteria were washed twice with PBS, and resuspended in PBS to 3 × 10^8 cells/ml. 1 ml of living bacteria was injected intraperitoneally into outbred NMRI mice. Plasma samples were taken 10 h after injection. Alternatively, mice were injected with the purified non-activated SCP (0.1–0.5 mg), and plasma samples were taken 60, 150, and 300 min after injection. For inactivation of SCP, 0.5 mg of the enzyme was mixed with 0.2 mg Z-Leu-Val-Gly-CHN_2 prior to injection. To monitor the cleavage of kininogen, 1 μl of plasma was run on SDS-PAGE followed by Western blotting with antibodies against bradykinin (α-BK).

Quantification of SCP in Mouse Plasma. One μl of plasma samples from mice injected with SCP was run on SDS-PAGE and transferred onto nitrocellulose. The enzyme was visualized by immunostaining using antibodies against SCP. To obtain semiquantitative estimates of the SCP amounts in plasma samples, purified SCP (3–100 ng) was processed as described above and used as a standard.

Results

Streptococcal Cysteine Proteinase Is Not Inhibited by H-Kininogen. The streptococcal cysteine proteinase (SCP) cleaves surface proteins of *S. pyogenes* strain AP1 (9). One of its target structures, the streptococcal M1 protein, specifically binds kininogens (16) the major cysteine proteinase inhibitors of human plasma. These observations prompted the notion that kininogens bound to the bacterial surface might regulate the proteolytic activity of SCP. We therefore tested the effect of H-kininogen on the hydrolysis of a chromogenic peptide substrate by SCP. Unexpectedly, H-kininogen had no inhibitory effect on the amidolytic activity of SCP (not shown), whereas the synthetic cysteine proteinase inhibitor E-64, efficiently blocked the SCP activity in the same assay. We therefore asked the question whether H-kininogen serves as a substrate—rather than an inhibitor for SCP.

SCP Degrades H-Kininogen. When we analyzed the reaction mixture of H-kininogen and SCP by SDS-PAGE we found that SCP rapidly and almost completely degraded H-kininogen (Fig. 2). To follow the breakdown of H-kininogen by SCP, and to identify potential cleavage products such as the biologically active kinin peptides, we employed Western blotting and immunoprinting of the reaction mixtures. We used polyclonal antibodies directed to the kinin sequence of nine residues located in domain D4 of H-kininogen, and monoclonal antibodies to the flanking domains, D3 and $D5_H$ (see Fig. 1). Fig. 3 shows three replicas of the SCP cleavage products of H-kininogen separated by SDS-PAGE and immunoprinted by a monoclonal antibody against the COOH-terminal part of domain D3 (HKH 15; Fig. 3 *A*), by a polyclonal antibody to bradykinin (α-BK; Fig. 3 *B*), and by a monoclonal antibody recognizing the NH_2-terminal part of domain $D5_H$ (HKL 9; Fig. 3 *C*), respectively. The immunoprints reveal a complex pattern of kininogen degradation products. The native H-kininogen of 105 kD is rapidly cleaved into fragments of 60–75 kD containing the D3 epitope (*A*), and into fragments of 45–70 kD comprising the $D5_H$ epitope (*C*). Initially the kinin epitope which is rapidly lost from the native kininogen of 105 kD, remains associated with a band of 60 kD that is also recognized by the anti-heavy chain antibody (*A*) but not by the anti-light chain antibody (*C*). This would indicate that the initial cleavage by SCP occurs at site(s) located distally of the bradykinin moiety, and therefore the bradykinin sequence remains attached to the heavy chain. Further proteolysis by SCP breaks down the kininogen heavy chain, most probably into its constituting domains (note that the various domains D1 through D3 of the kininogen

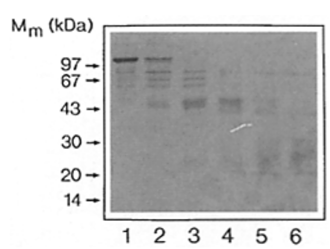

Figure 2. Cleavage of H-kininogen by the streptococcal cysteine proteinase (SCP). H-kininogen (30 μg) was incubated with 0.07 μg of SCP (molar ratio of 100:1). After 15 min (lane *2*), 30 min (lane *3*), 60 min (lane *4*), 120 min (lane *5*), or 180 min (lane *6*) of incubation aliquots of the reaction mixture (4 μg protein each) were separated by SDS-PAGE (10% vol/vol) under reducing conditions, followed by staining with Coomassie Brilliant Blue. For control H-kininogen incubated for 180 min in the absence of SCP was applied (lane *1*). Note that a small amount of the purified H-kininogen exists in its kinin-free two chain form. Standard molecular marker proteins were run simultaneously (not shown); their relative positions are indicated on the left.

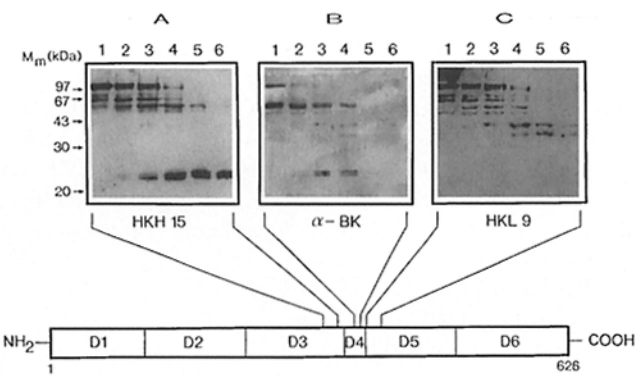

Figure 3. Immunoprint analysis of H-kininogen cleavage products. Aliquots from the reaction mixture of H-kininogen (30 μg) and SCP (0.07 μg) were removed after 15 min (lane *2*), 30 min (lane *3*), 60 min (lane *4*), 120 min (lane *5*), or 180 min (lane *6*) and separated by SDS-PAGE followed by electrotransfer onto nitrocellulose. For control native H-kininogen incubated for 180 min in the absence of SCP (*1*) was applied. Blots were incubated with HKH 15 antibody (*A*), α-BK antibodies (*B*), or HKL 9 antibody (*C*). Bound antibodies were visualized with peroxidase labeled anti-mouse or anti-rabbit immunoglobulins and the chemiluminescence technique. The relative locations of the antibodies target epitopes are indicated on the bottom; the domain designation is that of Fig. 1. Note that α-BK shows a higher affinity for kininogen fragments rather than for the uncleaved H-kininogen.

heavy chain are separated by protease-sensitive regions that expose the primary attack sites for many proteinases; 27). Accordingly, a prominent band of ~23 kD appears at the later stages of proteolysis representing domain D3 (*B*). A fraction of D3 still contains the COOH-terminal extension of bradykinin (*B*, lanes *3* and *4*) that is lost as proteolysis proceeds (⩾120 min). No shift in the apparent molecular mass of the D3 fragment is obvious (see *A*, lanes *3* to *6*) suggesting that only a minor peptide such as bradykinin is removed from the 23-kD fragment. Nevertheless, we sought to determine whether SCP releases authentic kinins from H-kininogen.

H-kininogen Cleavage Products Release Intracellular Ca^{2+} in Human Fibroblasts. To demonstrate the presence of biologically active kinins in the proteolytic digests we employed the fura-2/AM assay. This test system monitors the bradykinin B2 receptor-mediated release of Ca^{2+} from intracellular stores of human foreskin fibroblasts (25). Purified H-kininogen did not induce a Ca^{2+} release from human fibroblasts (Fig. 4 *A*); hence the starting product did not contain appreciable amounts of kinins. In contrast the reactions mixtures from the incubation of H-kininogen with SCP for 60 min (*C*) or 120 min (*D*) induced significant Ca^{2+} signals thus indicating the presence of biologically active kinins. The specificity of the assay was probed by preincubating the cells with the potent B2 receptor antagonist, HOE140, which completely abrogated the Ca^{2+} signal induced by the application of the kininogen breakdown products (data not shown). H-kininogen which had been incubated for 120 min in the absence of SCP induced no Ca^{2+} signal (data not shown); hence the kinin release was not due to a contaminating kininogenase associated with the starting material. Together these results demonstrate that SCP releases biologically active kinins from H-kininogen.

SCP Cleaves H-Kininogen in Plasma. To test whether SCP cleaves H-kininogen in its physiological environment, the streptococcal enzyme was added to plasma. After varying time points, aliquots were removed from the reaction mixture and subjected to Western blot analyses. Highly specific polyclonal antibodies to native H-kininogen (AS 88) and to bradykinin (α-BK) were applied to identify the kininogen cleavage products in the complex plasma mixture. Fig. 5 *A* demonstrates that the endogenous H-kininogen present in human plasma is partially degraded after 15 min, and almost completely split after 30 min of incubation with SCP. Be-

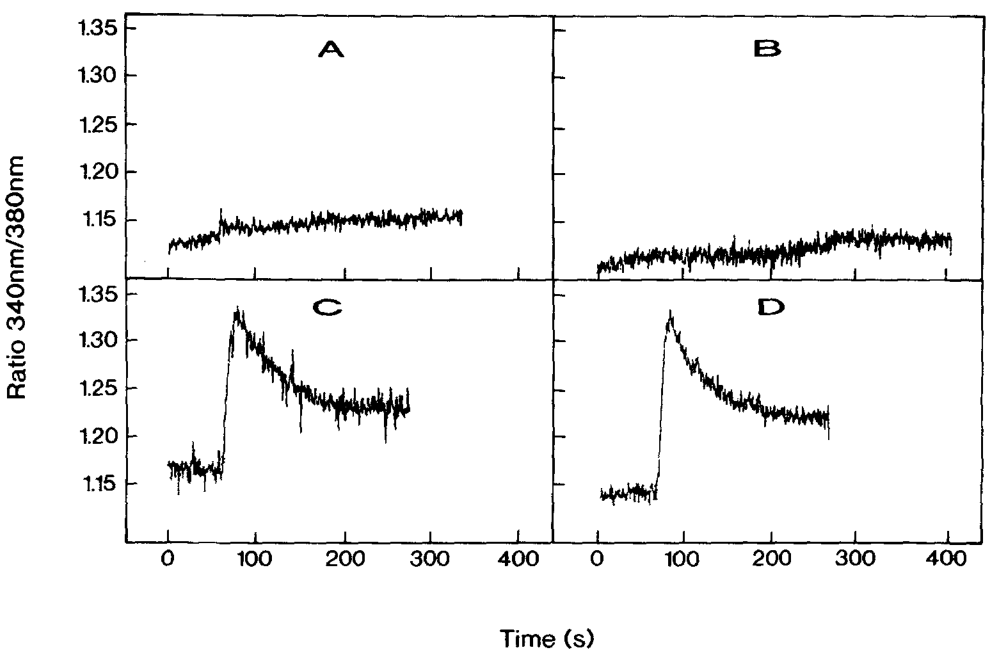

Figure 4. Ca^{2+} release from intracellular stores induced by H-kininogen cleavage products. Confluent human fibroblasts loaded with fura-2 were incubated with untreated H-kininogen (*A*), and H-kininogen cleaved by SCP for 30 min (*B*), 60 min (*C*), or 120 min (*D*) at 37°C. The intracellular Ca^{2+} release was measured as the ratio of fluorescence at excitation wavelengths of 340 nm and 380 nm, respectively.

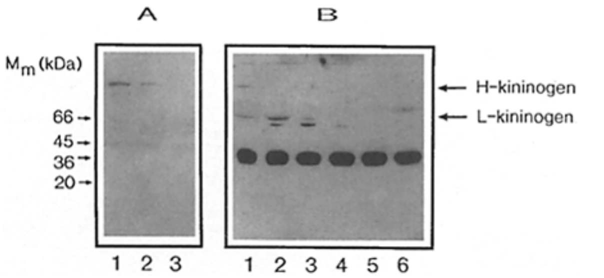

Figure 5. H-kininogen cleavage in plasma. Human plasma (100 μl) was incubated with 3.2 μg of SCP. Samples were taken after 15 min (lane *2*), 30 min (lane *3*), 45 min (lane *4*), 60 min (lane *5*), or 90 min (lane *6*) of incubation and separated by SDS-PAGE followed by the transfer of the proteins onto nitrocellulose and immunostaining by antibodies against native H-kininogen (AS88; *A*) or to BK (α-BK; *B*). For control, plasma was incubated in the absence of SCP for 90 min (lane *1*). The relative positions of the human plasma kininogens are marked on the right.

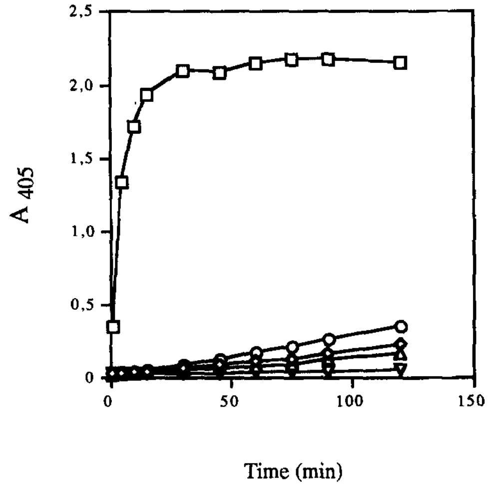

Figure 6. Time course of prekallikrein activation by various proteinases. Plasma prekallikrein was incubated for 1 h with factor XIIa in a molar ratio of 100:1 (□) or with SCP in molar ratio of 100:1 (◇) and 10:1 (○). At the indicated time points aliquots of the reaction mixtures were removed, and their amidolytic activity tested by a chromogenic substrate assay (H-D-Pro-Phe-Arg-pNA). For control, prekallikrein incubated in the absence of SCP (△), or SCP alone (▽) were tested.

cause the antiserum (AS88) is primarily directed to immunodominant epitopes of the H-kininogen light chain (20) it poorly cross-reacts with L-kininogen which is seen as a faint band of 66 kD (*A*, lane *1*; see *B*, lane *1*). The α-BK antibodies reacted weakly with the native forms of H-kininogen and L-kininogen, respectively (*B*, lane *1*). After 15 min of incubation a strong immunoreactivity at 66 kD is visible which likely corresponds to a kinin-containing fragment representing the kininogen heavy chain including the bradykinin epitope (note that the cleavage of a scissile bond flanking the kinin segment results in a major conformational change of the kininogen molecule and a concomitant exposure of the bradykinin epitope). Under the conditions of our experiment SDS-PAGE does not resolve the putative fragment and L-kininogen because the proteins differ only by 36 residues. After 15 min of SCP proteolysis a smaller fragment of ~60 kD is recognized by the anti-bradykinin antibodies (*B*, lane *2*). This latter fragment which peaks at 30 min (*B*, lane *3*) and fades away after prolonged incubation is likely to represent a degradation product of the kininogen heavy chain with bradykinin still attached to its carboxy terminus. Unlike the former band, i.e., heavy chain comprising the bradykinin epitope, the latter band, presenting a putative heavy chain degradation product, is not observed when kininogen is split by its physiological processing enzyme, plasma kallikrein (20). The prominent 45-kD band that occurs throughout the entire incubation procedure (Fig. 5 *B*) is likely to be a staining artefact of the α-BK antibodies when plasma is used; we did not observe such an immunoreactivity with purified H-kininogen (see Fig. 3). We could not detect any significant kininogen degradation in plasma that was incubated in the absence of SCP (data not shown). Together these data suggest that SCP degrades kininogen both in an isolated system and in complex mixtures such as plasma, and that the rapid loss of kinin immunoreactivity reflects the liberation of the hormone by SCP.

SCP Does Not Activate Purified Plasma Prekallikrein. Under physiological conditions, the kinin release from kininogens is mediated by activated plasma kallikrein. Due to a reciprocal activation factor XII converts the zymogen, prekallikrein, to the active enzyme, α-kallikrein. Hence, activation of plasma prekallikrein by SCP may explain at least in part the observed release of kinins from H-kininogen (see above). To test this possibility, prekallikrein isolated from human plasma was incubated with purified SCP, and followed by SDS-PAGE demonstrating that prekallikrein was rapidly processed by the streptococcal enzyme (data not shown). The resultant cleavage products were tested for their amidolytic activity in chromogenic assays using the *p*-nitroanilide derivative of the tripeptide, H-D-Pro-Phe-Arg. Prekallikrein cleavage products generated by varying concentrations of SCP did not reveal significant amidolytic activity (Fig. 6); likewise prekallikrein or SCP alone had no activity. In contrast prekallikrein activation by factor XIIa resulted in the progressive activation of the zymogen. Because SCP is unable to activate prekallikrein under the conditions of our experiment, we conclude that the bacterial enzyme is likely to act directly on kininogen present in human plasma without prior activation of a physiological kininogenase. This notion is supported by the observation that kininogen degradation products are formed by SCP that do not occur in the kallikrein-mediated processing cascade.

SCP Generates Kinins from Plasma Kininogens. Our proteolysis experiments demonstrated that biologically active kinins are released from H-kininogen by SCP in a purified system. We therefore asked the question whether SCP may liberate kinins from kininogens also in a complex environment such as the plasma. To this end we incubated human plasma with purified SCP for 2 h and tested aliquots of the reaction mixture after varying time periods. A competitive ELISA was employed and Fig. 7 demonstrates that SCP release kinins in a time-dependent manner. After 120 min of

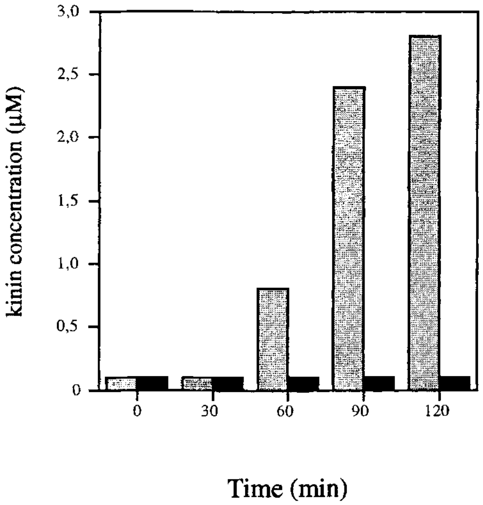

Figure 7. Kinin generation by SCP in plasma followed by ELISA. Human plasma (100 µl) was incubated with 3.2 µg of SCP (▩), aliquots of the reaction mixture were removed at the time points indicated, and assayed for their kinin concentration by a competitive ELISA. For control plasma was incubated under identical conditions except that SCP was omitted (■). Note that the lower detection limit for bradykinin is approximately 10^{-7} mol/l of plasma.

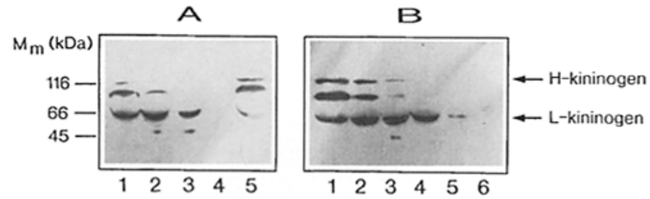

Figure 8. Cleavage of plasma kininogens by SCP in vivo. (*A*) Mice were injected i.p. with 0.5 mg of purified SCP. Plasma samples were drawn from the animals after 60 min (lane *2*), 150 min (lane *3*), and 300 min (lane *4*). Alternatively, 0.5 mg SCP mixed with 0.2 mg Z-Leu-Val-Gly-CHN_2 was injected, and a plasma sample from this mouse was taken 300 min after injection (lane *5*). For control, plasma from a mouse injected with PBS alone was used (lane *1*). 1 µl of each sample was separated by SDS-PAGE, transferred to nitrocellulose and immunostained with antibodies to bradykinin (α-BK). The relative positions of the marker proteins are given to the left, and those of the mouse plasma kininogens are indicated to the right. (*B*) Mice were injected (i.p.) with PBS alone (lane *1*), 0.1 mg of SCP (lane *2*), 0.2 mg of SCP (lane *3*), 0.3 mg of SCP (lane *4*), 0.4 mg of SCP (lane *5*), or 0.5 mg of SCP (lane *6*). Plasma samples were taken 300 min after injection.

incubation, the kinin concentration of samples had leveled off at 2.8 µM which almost approaches the theoretically releasable concentration of bradykinin in human plasma of 3.5 µM. Thus, approximately 0.9 µM H-kininogen and 2.6 µM L-kininogen are present in human plasma (28). No release of kinins was found in controls where plasma was incubated without SCP. These results demonstrate that SCP-induced cleavage of kininogen in plasma is combined with the release of kinins.

SCP Cleaves Kininogens In Vivo. To test whether SCP also processes kininogens in vivo, we injected purified SCP into the peritoneal cavity of mice. Two types of experiments were performed. In the first set of experiments, lethal doses of SCP (0.5 mg per animal) were administrated intraperitoneally, and plasma samples from these animals were taken 60 min, 150 min, and 300 min after injection (Fig. 8 *A*). For control, 0.5 µg SCP that had been inactivated by the specific inhibitor Z-Leu-Val-Gly-CHN_2 (4) was injected i.p. into mice, and plasma samples were withdrawn after 300 min. In a second set of experiments, varying amounts of SCP (0.1–0.5 mg) were injected i.p., and plasma samples were taken 300 min thereafter (Fig. 8 *B*). Kininogen degradation in plasma was detected by Western blotting, using antibodies to bradykinin. Three immunoreactive band of 66, 80, and 110-kD were detected in plasma of mice that had been treated with vehicle only; the upper 110-kD band and the lower 66-kD band correspond to H- and L-kininogen, respectively. The intermediate band of 80 kD may correspond to a modified form of mouse L-kininogen, ir-kininogen, that has recently been described in mouse fibroblasts (29). Plasma of mice that had been injected with SCP 60 min prior to bleeding completely lacked the immunoreactive H-kininogen band of 110 kD. After 150 min most of the plasma kininogens had been degraded, and after 300 min no kininogen fragments were detectable. By contrast the majority of plasma kininogens from animals that had been injected with the enzyme-inhibitor complex remained intact.

A dose-dependent effect of SCP on plasma kininogen degradation was found when we injected increasing amounts of SCP (Fig. 8 *B*). Even at lowest enzyme amounts (0.1 and 0.2 mg) a significant fraction of plasma kininogens was found to be degraded. At high SCP amounts (≥0.4 mg) hardly any kinin-containing kininogen fragments or fragments thereof were detectable. From semi-quantitative Western blot analyses we judged the plasma concentration of SCP to be in the range of 3–25 µg/ml of plasma dependent on the amount of injected enzyme and the time elapsed after injection (Table 1).

Alternatively, living streptococci of strain AP 1 were injected i.p.. Plasma samples were drawn then from the animals 8 h after injection, and analyzed by SDS-PAGE (Fig. 9).

Table 1. *Quantification of SCP in Mouse Plasma*

Amount of SCP administered (i.p.)	Time of the administration	Plasma concentration*
mg	*min*	*µg/ml*
0.5	60	12
0.5	150	20
0.5	300	25
0.1	300	2
0.2	300	12
0.3	300	12
0.4	300	20

*The SCP plasma concentration was judged from Western blots using purified SCP as the standard.

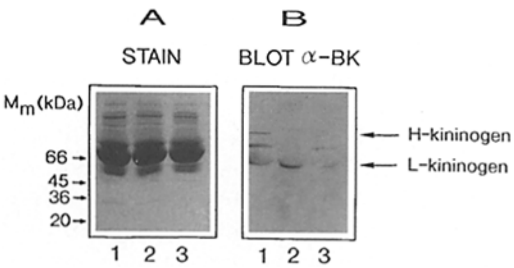

Figure 9. Cleavage of plasma kininogens by *S. pyogenes* in vivo. Mice were injected i.p. with 0.5 mg of purified SCP or with living *S. pyogenes* bacteria (3 × 10^8 cells) diluted in 0.5 ml PBS. (*A*) Plasma samples (1 μl each) from animals injected with PBS alone (lane *1*), with purified SCP (lane *2*), or with *S. pyogenes* bacteria (lane *3*) were run on SDS-PAGE and stained with Coomassie Brilliant Blue. (*B*) An identical replica on nitrocellulose was probed with antibodies to bradykinin (α-BK). Standard molecular marker proteins were run simultaneously (not shown); their relative positions are indicated on the left. The positions of mouse kininogens are marked.

Coomassie Brilliant Blue staining showed no apparent difference between normal mouse plasma and plasma samples from mice injected with SCP or AP 1 bacteria demonstrating that the overall protein composition of plasma was unchanged (Fig. 9 *A*). In the corresponding Western blots, SCP was detected in the plasma of mice given 0.5 mg of the enzyme i.p., but not in the plasma of mice infected with AP 1 bacteria, indicating that the concentration of SCP was lower in the latter experimental setting (data not shown). This observation may also explain why kininogens were not completely degraded in these animals (see Fig. 8 *B*). Immunoprinting of the plasma samples with α-BK antibodies revealed that native H-kininogen was completely absent from the plasma of mice treated with SCP as evidenced by the kinin immunoreactivity. Furthermore, kininogen concentrations were considerably though not completely reduced in the plasma of mice infected with *S. pyogenes* of the AP 1 strain (Fig. 9 *B*). These findings demonstrate that kininogens are also degraded by SCP in vivo, most likely under the release of kinins. For control we used AP 74 bacteria, the only strain of *S. pyogenes* that we have found not to produce SCP (Cooney, J., C. Liu, and L. Björck, manuscript in preparation). No significant decrease of kininogens was seen in plasma of mice treated with the same protocol as above except that AP 74 bacteria were used (not shown), thus underlining the specific role for SCP in kininogen turnover and kinin release. Together, these data demonstrate that purified SCP or SCP secreted by *S. pyogenes*, cleaves kininogens in vivo under the release of kinins.

Discussion

In recent years several lines of evidence have suggested a pathogenetic role for extracellular microbiological cysteine proteinases. Such enzymes appear to be involved in host colonization, tissue invasion, evasion of host defense mechanism, and modulation of immunological and inflammatory responses (for a review, see reference 30). Moreover, experimental data have suggested that a cysteine proteinase of *Trypanosoma cruzi* could be used as a target for immunoprophylaxis (31, 32).

SCP was the first prokaryotic cysteine proteinase to be isolated (2), and in these early studies the enzyme attracted attention by its capacity to destroy the type-specific M protein of *S. pyogenes*, a major virulence determinant of these bacteria (see M protein review, reference 33). During the following decades the protein chemical and enzymatic properties of SCP were described in numerous investigations, especially by Elliott and Liu and their coworkers (for a review, see reference 34). *S. pyogenes* produces the three erythrogenic exotoxins A, B and C, and out of these exotoxin B was shown to be identical to SCP (3, 35). In a culture of *S. pyogenes*, SCP first appears in the growth medium as an inactive zymogen of 40 kD that is transformed into the active proteinase (28 kD) by limited proteolysis or by autocatalysis under reducing conditions (36). Interestingly, SCP is also found in its active form within the streptococcal cell (37) suggesting that the enzyme has also intracellular functions. This notion is supported by the finding that specific blockage of cysteine proteinase activity blocks *S. pyogenes* growth in vitro (4) implying that cysteine proteinases such as SCP may serve essential functions also in prokaryotic cells. Several observations indicate that the secreted form of SCP contributes to the virulence of *S. pyogenes*. For example the proteinase degrades abundant extracellular matrix proteins like fibronectin and vitronectin (8), and it activates the pro-inflammatory cytokine interleukin-1β (7). In addition, SCP releases biologically active fragments from various surface proteins of *S. pyogenes* (9). One of these, a fragment of streptococcal C5a peptidase, was found to block C5a-mediated granulocyte migration (10). These and other findings (38) support the notion that SCP is a major virulence determinant.

The starting point for our present investigation into a possible link between SCP and kininogens was the demonstration that most strains of *S. pyogenes* bind human plasma kininogens specifically and tightly through their surface-associated antiphagocytic M-protein (16). The finding that kininogens are attached to the bacterial surface raised the question whether these potent cysteine proteinase inhibitors might regulate the proteolytic activity of secreted SCP. Unexpectedly, our experiments demonstrated that H-kininogen is unable to inhibit SCP; the reason for this failure is presently unknown. Rather H-kininogen was a substrate for SCP, and the resultant cleavage pattern suggested that kinins might have been released from kininogen by the specific action of SCP, an assumption that was subsequently verified. Hence SCP is an efficient kininogenase which releases the pro-inflammatory kinins in solution. We have not tested yet whether surface-bound kininogens are also processed by SCP, but the fact that kinins are released from neutrophil-bound kininogens by kallikreins (39) clearly points to such a possibility.

The recruitment of kininogens from human body fluids by M-protein at the streptococcal surface will lead to the local accumulation of the kinin precursor molecules in infected tissues. If SCP secreted by the bacteria cleaves these kininogen molecules, a local burst of kinins will cause increased vascular permeability. Such a sequence of events would promote a flow of nutrients into the site of infection and at the same time enhance the spreading of the infection via facilitated extravasation. This hypothetical scenario is supported by the observation that SCP secretion is dependent on environmental factors such as pH (34). Notably the pH is low in the center of suppurative streptococcal infections (40), and most strains of *S. pyogenes* produce excessive amounts of SCP (10–150 mg/L of growth medium) when grown at pH 5.5–6.0.

In severe cases of sepsis, hypovolemic hypotension is a prominent and clinically important finding that is caused by the leakage of plasma into the extravascular space (41). The rapid and efficient cleavage of kininogens to kinins in mouse plasma following the administration of SCP or living *S. pyogenes* bacteria is the major finding of this study. It indicates that a general and massive release of kinins could take place in severe streptococcal infections, such as sepsis and streptococcal toxic shock syndrome. These conditions are characterized by raging fever, drop in blood pressure, and multiorgan failure (1, 41). In this context it is noteworthy that patients with low titers of antibodies to SCP in the acute phase are more likely to die in severe *S. pyogenes* infections (6), and that immunization of mice with SCP generates partial protection against *S. pyogenes* administrated i.p. (5). Furthermore, a single dosis of a tripeptide derivative which blocks the enzymatic activity of SCP (4) cures mice given an otherwise lethal dosis of this enzyme (Cooney, Liu, and Björck, in preparation). These and other data described above, underline the significance of SCP in the pathogenesis of streptococcal infections. The major and novel aspect of this work is that we have identified a potential downstream effector of SCP, i.e., kinins. Recruitment of host proteins and exploitations of their intrinsic properties by the parasite is a phenomenon that is probably common to many pathogenic bacteria. Our results also indicate that SCP and/or kinins could be therapeutical targets in hyperacute and severe *S. pyogenes* infections where treatment with antibiotics alone is insufficient. Specific inhibition of these potential mediators of shock could interrupt an otherwise fatal pathologic sequence.

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