Streptococcal Cysteine Proteinase Releases Kinins: a Novel Virulence Mechanism

By Heiko Herwald,* Mattias Collin,* Werner Müller-Esterl,† and Lars Björck*

From the *Department of Cell and Molecular Biology, Section for Molecular Pathogenesis, Lund University, S-221 00 Lund, Sweden; and †Institute of Physiological Chemistry and Pathobiochemistry, Johannes Gutenberg University at Mainz, Duesbergweg 6, D-55099 Mainz, Germany

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-CHN₂, 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.

*S. 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) 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.
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 to H-kininogen (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-CHN₂ 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 NaH₂PO₄, 10 mM Na₂HPO₄, 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. A aliquot (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-(1-3-transcarboxyoxiran-2-carbonyl)-1-leucyl]-agmatine (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 KH₂PO₄, 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.

**Ca²⁺ Release from Intracellular Stores.** Human foreskin fibroblasts (HF-15) on 10-mm diameter glass coverslips were grown to confluence 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 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, pentacetoxymethylester (fura-2/AM; Calbiochem Nova-biochem, San Diego, CA) in buffer A containing 0.04% (wt/vol) of the nonionic detergent pluronic F-127 (Calbiochem Nova-biochem) (25). The cells were washed twice with buffer A. The Hitachi F4500 fluorescence photometer was employed with the

![Figure 1](Image)

**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, D5₄/D₅₆ and D5₄, 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 expresses a cell binding site. The kinin segment is located in domain D4. Domain D5₄ of H-kininogen exposes a high-affinity cell binding site which is also used by streptococcal M protein. Domain D6₄ contains the overlapping binding sites for prekallikrein and factor X. The function of D5₄ 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 Ca²⁺ 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 Ca²⁺ from intracellular stores was followed for 300 s; the free intercellular Ca²⁺ 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) H₂O₂ in 100 mM citric acid, 100 mM Na₂HPO₄, 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 × 10⁸ cells/ml. 1 ml of living bacteria was injected intraperitoneally into outbred NMRI mice. Plasma samples 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 × 10⁸ 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 ng), 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-CHN₂ 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 D5H (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 NH₂-terminal part of domain D5H (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 D5H 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).](image-url)
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 Ca²⁺ 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 Ca²⁺ from intracellular stores of human foreskin fibroblasts (25). Purified H-kininogen did not induce a Ca²⁺ 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 Ca²⁺ 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 Ca²⁺ 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 Ca²⁺ 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. Ca²⁺ 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 Ca²⁺ release was measured as the ratio of fluorescence at excitation wavelengths of 340 nm and 380 nm, respectively.
cause the antiserum (AS88) is primarily directed to immuno
dominant 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-ki
ninogen 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 frag
ment 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 conforma
tional change of the kininogen molecule and a concomi
tant exposure of the bradykinin epitope). Under the condi
tions 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 pro
longed incubation is likely to represent a degradation prod
uct of the kininogen heavy chain with bradykinin still at
tached 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 incu
bated 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 ki
ninogens is mediated by activated plasma kallikrein. Due to a

SCP Generates Kinins from Plasma Kininogens. Our pro
teolysis 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 kininogen also in a complex environ
ment 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 re
lease kinins in a time-dependent manner. After 120 min of

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 na-
tive 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 posi-
tions of the human plasma kininogens are marked on the right.

Figure 6. Time course of prekallikrein activation by various protein-
ases. Plasma prekallikrein was incubated for 1 h with factor XIIa in a mo-
lar 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.
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-CHN₂ (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 could be 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.
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 blockade 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-1B (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 kininogen 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 \textit{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 \textit{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 \textit{S. pyogenes} infections (6), and that immunization of mice with SCP generates partial protection against \textit{S. pyogenes} administered 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 \textit{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.

We wish to thank our colleagues Drs. W. Machleidt and I. Asfälgs-Machleidt (Ludwig Maximilian University, Munich) for experimental help with the inhibitor assays; A. Maidhof and B. Welch (Johannes Gutenberg University, Mainz) for providing antisera; A. Horstmeyer (Mainz) for experimental help with the fura assay, U. Quitterer (University of Würzburg) and P. Åkesson (Lund University) for their critical comments on the manuscript, and B. Jörnson (Lund University) for preparing the figures.

This work was supported in part by The Swedish Medical Research Council (grant 7480), the Deutsche Forschungsgemeinschaft (Ma598/5.2), the Fonds der Chemischen Industrie (to W. Müller-Esterl), the Foundations of Kock and Österlund, and High Tech Receptor AB.

Address correspondence to Heiko Herwald, Department of Cell and Molecular Biology, Section for Molecular Pathogenesis, Lund University, P.O. Box 94, S-221 00 Lund, Sweden.

Received for publication 12 March 1996 and in revised form 28 May 1996.

**References**

1. Nowak, R. 1994. Flesh-eating bacteria: not new, but still worrisome. *Science (Wash. DC).* 264:1665.
2. Elliott, S.D. 1945. A proteolytic enzyme produced by group A streptococci with special reference to its effect on the type-specific M antigen. *J. Exp. Med.* 81:573–592.
3. Gerlach, D., H. Knoll, W. Köhler, J.H. Oezegowski, and V. Hribalova. 1983. Isolation and characterization of erythrogenetic oxins. V. Communication: identity of erythrogenetic toxin type B and streptococcal proteinase precursor. *Zentralbl. Bakteriol. Mikrobiol. Hyg.* A. 255:221–233.
4. Björck, L., P. Åkesson, M. Bohus, J. Trojnar, M. Abrahamsson, I. Olafsson, and A. Grubb. 1989. Bacterial growth blocked by a synthetic peptide based on the structure of a human proteinase inhibitor. *Nature (Lond).* 337:385–386.
5. Kapur, V., J.T. Maffei, R.S. Greer, L.L. Li, G.J. Adams, and J.M. Musser. 1994. Vaccination with streptococcal extracellular cysteine protease (interleukin-1 beta convertase) protects mice against challenge with heterologous group A streptococci. *Microb. Pathol.* 16:443–450.
6. Holm, S.E., A. Norby, A.-M. Bergholm, and M. Norgren. 1992. Aspects of patogenesis of serious group A streptococcal infections in Sweden 1988–1989. *J. Infect. Dis.* 166:31–37.
7. Kapur, V., M.W. Majesky, L.L. Li, R.A. Black, and J.M. Musser. 1993. Cleavage of interleukin 1 beta (IL-1 beta) precursor to produce active IL-1 beta by a conserved extracellular cysteine protease from *Streptococcus pyogenes.* *Proc. Natl. Acad. Sci. USA.* 90:7676–7680.
8. Kapur, V., S. Topouzis, M.W. Majesky, L.L. Li, M.R. Humrick, R.J. Hamill, J.M. Patrizi, and J.M. Musser. 1993. A conserved *Streptococcus pyogenes* extracellular cysteine protease cleaves human fibronectin and degrades vitronectin. *Microb. Pathol.* 15:327–346.
9. Berge, A., and L. Björck. 1995. Streptococcal cysteine proteinase releases biologically active fragments of streptococcal surface proteins. *J. Biol. Chem.* 270:9862–9867.
10. Wexler, D.E., D.E. Chenoweth, and P.P. Cleary. 1985.
Mechanism of action of the group A streptococcal C5a inactivator. *Proc. Natl. Acad. Sci. USA.* 82:8144–8148.

11. Wolf, B.B., C.A. Gibson, V. Kapur, I.M. Hussain, J.M. Musser, and S.I. Gonas. 1994. Proteolytically active streptococcal pyrogenic exotoxin B cleaves monocytic cell urokinase receptor and releases an active fragment of the receptor from the cell surface. *J. Biol. Chem.* 269:30682–30687.

12. Poon-King, R., J. Bannan, A. Viteri, G. Cu, and J.B. Zabriskie. 1993. Identification of an extracellular plasmin binding protein from nephritogenic streptococci. *J. Exp. Med.* 178:759–763.

13. Hall, J.M. 1992. Bradykinin receptors: pharmacological properties and biological roles. *Pharm. Ther.* 56:131–190.

14. Kerbiriou, D.M., and J.H. Griffin. 1979. Human High Molecular Weight Kinogen. *J. Biol. Chem.* 254:12020–12027.

15. Müller-Esterl, W., G. Rauth, F. Lottspeich, J. Kellermann, and A. Henschel. 1985. Limited proteolysis of human low-molecular-mass kininogen by tissue kallikrein. Isolation and characterization of the heavy and the light chains. *Eur. J. Biochem.* 149:15–22.

16. Ben Nasr, A.B., H. Herwald, W. Müller-Esterl, and L. Björck. 1995. Human kininogens interact with M protein, a bacterial surface protein and virulence determinant. *Biochem. J.* 305:173–180.

17. Salvesen, G., C. Parkes, M. Abrahamson, A. Grubb, and A.J. Barrett. 1986. Human low-M, kininogen contains three copies of a cystatin sequence that are divergent in structure and in inhibitory activity for cysteine proteinases. *Biochem. J.* 234:429–434.

18. Hasan, A.A., D.B. Cines, J. Zhang, and A.H. Schmaier. 1994. The carboxyl terminus of bradykinin and amino terminus of the light chain of kininogens comprise an endothelial cell binding domain. *J. Biol. Chem.* 269:31822–31830.

19. Kauffmann, J., M. Haasemann, S. Modrow, and W. Müller-Esterl. 1993. Structural dissection of the multidomain kininogens. Fine mapping of the target epitopes of antibodies interfering with their functional properties. *J. Biol. Chem.* 268:9079–9091.

20. Müller-Esterl, W., D. Johnson, G. Salvesen, and A.A. Barrett. 1988. Human kininogens. *Methods Enzymol.* 163:240–256.

21. Herwald, H., A.H.K. Hasan, J. Godovac-Zimmermann, A.H. Schmaier, and W. Müller-Esterl. 1995. Identification of an endothelial cell binding site on kinogen domain D3. *J. Biol. Chem.* 270:14634–14642.

22. Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)* 227:680–685.

23. Khyse-Andersen, J. 1984. Electroblotting of multiple gels: a simple apparatus without buffer tank for rapid transfer of proteins from polyacrylamide to nitrocellulose. *J. Biochem. Biophys. Methods.* 10:203–209.

24. Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA.* 76:4350–4354.

25. Quitterer, U., C. Schröder, W. Müller-Esterl, and H. Rehm. 1995. Effects of bradykinin and endothelin-1 on the calcium homeostasis of mammalian cells. *J. Biol. Chem.* 270:1992–1999.

26. Scott, C.F., E.J. Whitaker, B.F. Hammond, and R.W. Colman. 1993. Purification and characterization of a potent 70-kDa thiol lysyl-proteinase (Lys-gingivain) from *Porphyromonas gingivalis* that cleaves kininogens and fibrinogen. *J. Biol. Chem.* 268:7935–7942.

27. Vogel, R., I. Assafag Machleidt, A. Esterl, W. Machleidt, and W. Müller-Esterl. 1988. Proteinase-sensitive regions in the heavy chain of low molecular weight kininogen map to the inter-domain junctions. *J. Biol. Chem.* 263:12661–12668.

28. Müller-Esterl, W. 1987. Novel functions of kininogens. *Semin. Thromb. Hemostas.* 13:115–126.

29. Takano, M., K. Yokoyama, K. Yayama, and H. Okamoto. 1995. Murine fibroblasts synthesize and secrete kininogen in response to cyclic-AMP, prostaglandin E2 and tumor necrosis factor. *Biochim. Biophys. Acta.* 1265:189–195.

30. Travis, J., J. Potempa, and H. Maeda. 1995. Are bacterial proteinases pathogenic factors? *Trends Microbiol.* 3:405–407.

31. Eakin, A.E., M.E. McGrath, J.H. McKerrow, R.J. Fletterick, and C.S. Craik. 1993. Production of crystallizable cruzain, the major cysteine protease from *Trypanosoma cruzi*. *J. Biol. Chem.* 268:6115–6118.

32. Martinez, J., O. Campetella, A.C. Frasch, and J.J. Cazzulo. 1991. The major cysteine proteinase (cruzipain) from *Trypanosoma cruzi* is antigenic in human infections. * Infect. Immunol.* 59:4275–4277.

33. Fischetti, V.A. 1989. Streptococcal M protein: molecular design and biological behavior. *Clin. Microbiol. Rev.* 2:285–314.

34. Liu, T.-Y., and S.D. Elliott. 1965. Streptococcal proteinase. *J. Biol. Chem.* 234:285–314.

35. Hauser, A.R., and P.M. Schlievert. 1990. Identification of a cystatin sequence that are divergent in structure and in inhibitory activity for cysteine proteinases. *Biochem. J.* 234:429–434.

36. Liu, T.-Y., and S.D. Elliott. 1971. The Enzymes Vol. 3. P.D. Boyer, editor. Academic Press, New York. 609–639.

37. Hauser, A.R., and P.M. Schlievert. 1990. Nucleotide sequence of the streptococcal pyrogenic exotoxin type B gene and relationship between the toxin and the streptococcal proteinase precursor. *J. Bacteriol.* 172:4536–4542.

38. Liu, T.-Y., and S.D. Elliott. 1965. Streptococcal proteinase: the zymogen to enzyme transformation. *J. Biol. Chem.* 240:1138–1142.

39. Lo, S.S., S.M. Liang, and T.Y. Liu. 1984. Intracellular form of streptococcal proteinase: a clue to a novel mechanism of secretion. *Anal. Biochem.* 136:89–92.

40. Kellner, A., and T. Robertson. 1954. Myocardial necrosis in vitro and in vivo in chronic tonsillitis. *Z. Laryngol. Rhirol. Otol.* 49:391–397.

41. Parrillo, J.E. 1993. Pathogenetic mechanisms of septic shock. *N. Engl. J. Med.* 328:1471–1477.