Absorption of kininogen from human plasma by *Streptococcus pyogenes* is followed by the release of bradykinin

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H-kininogen (high-molecular-mass kininogen, HK) is the precursor of the vasoactive peptide hormone bradykinin (BK). Previous work has demonstrated that HK binds to *Streptococcus pyogenes* through M-proteins, fibrous surface proteins and important virulence factors of these bacteria. Here we find that M-protein-expressing bacteria absorb HK from human plasma. The HK bound to the bacteria was found to be cleaved, and analysis of the degradation pattern suggested that the cleavage of HK at the bacterial surface is associated with the release of BK. Moreover, addition of activated plasma prekallikrein to bacteria preincubated with human plasma, resulted in BK release. This mechanism, by which a potent vasoactive and proinflammatory peptide is generated at the site of infection, should influence the host–parasite relationship during *S. pyogenes* infections.

INTRODUCTION

M-proteins are fibrous, hair-like structures expressed at the surface of *Streptococcus pyogenes* (see [1,2]). They are regarded as major virulence determinants due to their anti-phagocytic property, and exist in more than 80 different serotypes. A role for M-protein in fibrinolysis was suggested by the observation that some M-proteins bind plasminogen [3]. Moreover, recent studies have demonstrated that M-proteins of most serotypes have affinity for kininogens, especially high-molecular-mass kininogen (H-kininogen, HK) [4]. In HK the binding of M-protein was localized to regions in the light chain, a portion of HK that mediates contact-phase activation (for kininogen see [5]). HK is a multi-functional protein that, together with the other contact-phase factors (factor XI, factor XII and plasma prekallikrein), participate in molecular events leading both to the proteolytic release of bradykinin (BK) from HK, and to the activation of the endogenous blood coagulation cascade.

S. pyogenes is an important human pathogen causing common suppurative infections, such as pharyngitis and skin infections, and also hyperacute and life-threatening toxic conditions, as well as serious post-infectious conditions (rheumatic fever and glomerulonephritis). BK is a potent proinflammatory peptide inducing fever and pain, increased vascular permeability and smooth-muscle contraction. With this background, the previous observation that HK is bound to the surface of *S. pyogenes* through M-protein, raised the question of whether this interaction represents a first step leading to the release of BK from surface-bound HK, and the subsequent triggering of inflammatory responses. The present investigation is focused on this question, and the results imply that the contact-phase system could play a role in streptococcal pathogenicity.

MATERIALS AND METHODS

Bacterial strains and culture conditions

S. pyogenes strains AP1 (40/58) and AP46 (1/59), expressing the M1 and M46 proteins respectively and the M negative strain AP74 (30/50) were from the Institute of Hygiene and Epidemiology (Prague, Czech Republic). Bacteria were grown in Todd Hewitt broth (Difco) at 37 °C for 16 h, washed twice in PBS (0.15 M NaCl/0.02 M phosphate, pH 7.4) containing 0.02 % (w/v) NaN_3 (PBSA), and resuspended in PBSA to 2×10^{10} cells/ml. Cells from this stock suspension were used in plasma absorption experiments and kallikrein digestion assays.

Proteins and labelling of proteins

Kininogens were purified from human plasma as previously described [5]. The monoclonal antibody HKL16, directed to an epitope in domain 6 (domain $D6_H$) of the light chain of HK, was raised in mouse and affinity purified on Protein A–Sepharose [6]. The monoclonal antibody HKL19, also directed against the $D6_H$ domain of HK, has been described previously [6]. Prekallikrein was isolated from human plasma [7]. Human factor XI was kindly given by Dr. J. Meijers, Department of Hematology, University of Utrecht, The Netherlands. Human factor XII was from Enzyme Research Laboratories, South Bend, IN, U.S.A. Recombinant M1 and M46 proteins were produced as described previously [3,8].

Plasma absorption experiments

The plasma absorption experiments were performed as described previously [9,10]. Fresh human plasma was mixed with proteinase inhibitors (Sigma) to a final concn. of 2 mM aprotinin/0.1 mM di-isopropyl fluorophosphate/1 mM soya bean trypsin inhibitors/0.1 mM PMSF/5 mM benzamidine chloride. Bacteria (2×10^{10} cells/ml) suspended in 1 ml PBST [PBS containing 0.05 % (v/v) Tween 20] were incubated with 1 ml of human plasma for 60 min at 37 °C. The cells were pelleted and washed 3 times with 10 ml of PBST. To elute the absorbed proteins, the bacteria were incubated for 5 min with 0.5 ml of 30 mM HCl, pH 2.0. The bacteria were pelleted and the supernatant was immediately buffered with 50 μl of 1 M Tris, concentrated to 250 μl (with a buffer change to PBS) by ultrafiltration on an Amicon Centricon-30 (Amicon, Beverly, MA, U.S.A.). Protein solution (20 μl) was analysed by SDS/PAGE and immunoprint experiments using the monoclonal antibody HKL16.

Abbreviations used: HK, H-kininogen; BK, bradykinin.

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ELISA

The Sandwich ELISA technique was performed as detailed previously [5,6,11] with modification as follows: microtitre plates (Immunolon, Dynatech) were coated with 1 μg/ml of the monoclonal antibody HKL19 in 15 mM $NaHCO_3/Na_2CO_3$ buffer, pH 9.6, containing 1.5 mM NaN_3 by overnight incubation at 4 °C. The plates were washed 5 times with 20 mM phosphate buffer, pH 7.4, containing 150 mM NaCl and 0.05 % (w/v) Tween 20 (PBST). Serial doubling dilutions of HK standards (starting at a concn. of 2 μg/ml) or samples were prepared in PBST containing 2 % (w/v) BSA, and 200 μl were added to the coated wells in duplicate. After incubation for 1 h at 37 °C and washing as above, 200 μl of a polyclonal anti-HK sheep IgG, diluted 1:1000 in PBST containing 2 % BSA, were applied per well, followed by incubation for 2 h at 37 °C. The plates were extensively washed (see above) and 200 μl of a peroxidase-conjugated anti-sheep secondary antibody was added and incubated for 2 h at 37 °C. Finally, after washing as above, 200 μl of 0.1 % (w/v) ABTS [2,2′-azino-bis(3-ethyl-2,3-dihydrobenzthiazoline)-6-sulphonate]/0.05 % (v/v) H_2O_2 in 0.1 M citric acid/0.1 M $NaHPO_4$ was added to each well and the plates were incubated for 30 min at 37 °C in the dark. The change in absorbance at 405 nm was measured, and the amounts of HK in the samples were determined using the HK standard curve.

Electrophoresis and electroblotting

SDS/PAGE was performed as described previously [12]. Proteins in eluates from absorption experiments with bacteria were separated on gels of 10 % (w/v) total acrylamide with 3 % (w/v) bisacrylamide. Before loading, samples were boiled for 3 min in sample buffer containing 2 % (w/v) SDS and 5 % (v/v) β-mercaptoethanol. For Western blotting analysis, proteins were transferred to PVDF membranes (Immobilon, Millipore) by electroblotting from gels as described previously [13] using a Trans Blot semi-dry transfer cell (Bio-Rad, Irvine, CA, U.S.A.).

Determination of affinity constants

Binding kinetics were determined by surface plasmon resonance spectroscopy using a BIAlite biosensor system (Pharmacia Biosensor AB, Freiburg, Germany). HK was immobilized on research-grade CM5 sensor chips in 10 mM sodium acetate, pH 4.5, using the amine coupling kit supplied by the manufacturer [14]. All measurements were carried out in Hepes-buffered saline that contained 10 mM Hepes, pH 7.4, 150 mM NaCl, 3.3 mM EDTA and 0.005 % (v/v) Surfactant P.20 (Pharmacia Biosensor AB). Analyses were performed at 25 °C and at a flow rate of 10 μl/min. To calculate affinity constants, 30 μl of streptococcal M1 protein and M46 protein were applied in a serial dilution (starting concn. 100 μg/ml) followed by injection of buffer alone (30 μl). In addition, 30 μl of plasma kallikrein samples (doubling dilution; starting concn. 25 μg/ml) were assayed for comparison. Surfaces were regenerated with 30 μl of 10 mM HCl at a flow rate of 10 μl/min. The kinetic data were analysed by the BIA evaluation 2.0 program (Pharmacia Biosensor AB). Alternatively, 30 μl of M1 protein (50 μg/ml) and plasma kallikrein (10 μg/ml) alone or in combination were added and the amount of bound protein was determined, expressed in resonance units (RU) according to the manufacturer's descriptions.

Immunoprint analysis of HK and its fragments

Immunoprint analysis was performed as described previously [9]. Briefly, the PVDF membranes were blocked in PBST containing 5 % (w/v) non-fat dry milk for 20 min at 37 °C [15], washed three times with PBST for 5 min and incubated with antibodies against HK (in the blocking buffer) for 30 min at 37 °C. After washing, the sheets were incubated with peroxidase-conjugated secondary antibodies for 30 min at 37 °C. Secondary antibodies were detected by the chemiluminescence method [15,16]. Autoradiography was done at room temperature for 1–2 min using Kodak X-Omat S films and Cronex Extra Plus intensifying screens.

Kallikrein digestion assay and quantification of released BK

Plasma prekallikrein was activated to plasma kallikrein by cleavage with activated factor XIIa as described previously [17]. Briefly, plasma prekallikrein was mixed with factor XIIa in a molar ratio of 10:1 in PBS, pH 7.4, and incubated for 2 h at 37 °C and immediately used for proteolysis experiments (see below). Bacteria (4×10^{10} cells) in 0.5 ml of PBST were incubated with 1.5 ml of human plasma diluted 2:1 in PBST containing protease inhibitors (see Plasma absorption experiments section above). After incubation for 1 h at 37 °C the cells were washed 3 times in 10 ml of PBST and resuspended in 0.4 ml of kallikrein assay buffer/0.15 M Tris/HCl (pH 8.3) [7]. Bacteria were mixed with 100 μl of plasma kallikrein (giving a final enzyme concn. of 0.02 μg/ml) in an Eppendorf tube and incubated for 10 min at 37 °C. An equal amount of bacterial suspension was incubated, as a control, with 10 μl of kallikrein assay buffer without kallikrein. The incubation was ended by adding 0.4 ml of 60 mM HCl. Cells were pelleted and the supernatants were immediately buffered by adding 100 μl of 1 M Tris and ultrafiltered through an Amicon Centricon-10 filter (Amicon) to separate free BK from kininogen fragments. The resulting filtrates were analysed for their BK content by solid-phase radioimmunoassay using the MARKIT-M bradykinin TM kit (Dainippon Pharmaceutical, Osaka, Japan).

RESULTS

HK is absorbed from human plasma by M-protein-expressing *S. pyogenes* bacteria

M-proteins are known to interact with a number of different plasma proteins, including fibrinogen, plasminogen, immunoglobulins, albumin, and factor H and C4-binding protein of the complement system (see [2]). This raises the question of whether the interaction between HK and M-protein can take place in the presence of these other M-protein-binding plasma proteins, especially as proteins like albumin, IgG and fibrinogen occur at much higher concentrations than HK (HK represents approx. 0.15 % of the total protein content in plasma). To address this question, two strains of *S. pyogenes*, expressing M1 and M46 protein respectively, were incubated with fresh human plasma. Following extensive washing, proteins bound to the bacterial surface were eluted and the amount of HK was determined by ELISA. In a representative experiment the amounts of HK eluted from M1 and M46 bacteria were 63.6 and 123.6 pmol/10^{12} bacteria respectively, whereas an M-protein-negative mutant strain (AP74) absorbed HK at background level (below 10 pmol/10^{12} bacteria). The results demonstrate that HK, even in a plasma environment, can interact with M-protein-expressing *S. pyogenes* bacteria.

Analysis of the binding of M-proteins to HK suggests that HK is accumulated at the streptococcal surface

Using surface plasmon resonance spectroscopy, the binding kinetics between HK and the M1 and M46 proteins were determined and compared with the interaction between HK and plasma prekallikrein. In these experiments different amounts of

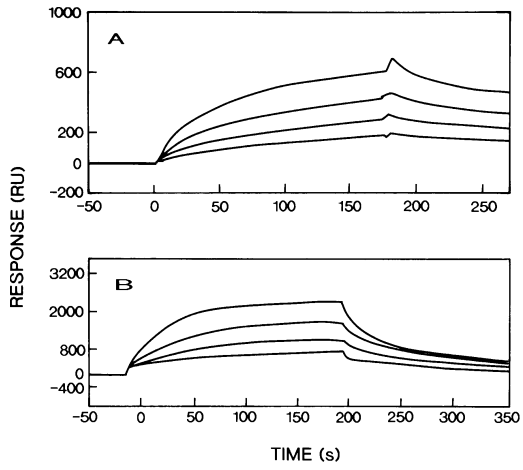

Figure 1 Analysis of the interactions between HK and M1 protein, and HK and plasma prekallikrein

Overlay plots of the binding of M1 protein (**A**) and plasma prekallikrein (**B**) to immobilized HK using plasmon resonance spectroscopy are shown. Increasing concentrations of M1 protein (12.5, 25, 50 and 100 μg/ml) or plasma prekallikrein (3.13, 6.25, 12.5 and 25 μg/ml) were applied for 3 min each during the association phase. Dissociation of bound proteins was measured (expressed in resonance units, RU) following injection of buffer alone.

Table 1 Affinity rates and dissociation constants for the interactions between immobilized HK and M1 protein, M46 protein or plasma prekallikrein

Values are means $\pm$ S.E.M. from at least three different experiments.

Ligand	$10^3 \times$ Association rate ($s^{-1} \cdot M^{-1}$)	$10^{-3} \times$ Dissociation rate (s^{-1})	$10^{-8} \times$ Dissociation constant (M)
M1 protein	5.5 ± 0.8	3.7 ± 0.7	68.3 ± 15.7
M46 protein	9.5 ± 2.1	2.3 ± 0.3	24.8 ± 5.9
Prekallikrein	496 ± 128	18.3 ± 5.4	3.8 ± 1.3

purified M-proteins or plasma prekallikrein were applied and left to interact with immobilized HK to the level of saturation. Figure 1 shows typical sensorgrams obtained between HK and M1 protein (Figure 1A) and plasma prekallikrein (Figure 1B). The results obtained for HK-M46 (not shown) were similar to those for HK-M1 shown in Figure 1(A). On the basis of these experiments, dissociation and association rates were calculated, which, when divided, give the dissociation constants (Table 1). The data demonstrate that the dissociations of M1 and M46 proteins occur more slowly than their associations, permitting an accumulation of HK at the bacterial surface. It can also be noted that the HK affinity is higher for M46 than for M1 protein. In plasma, prekallikrein circulates in complex with HK. As shown in Figure 1(B) and Table 1, both the association and dissociation rates are high for the interaction between these proteins. However, different data suggest that the binding of M-proteins to HK is not disturbed by the interaction between plasma prekallikrein and HK. Thus, prekallikrein interacts with HK through the $D6_H$ domain, residues 569–595 [17], whereas the binding site for M1 protein in HK is in domain $D5_H$, residues 479–496 [9]. Moreover, when a sample containing both M1 protein and prekallikrein was applied to an HK-coupled sensor chip, a binding of 909 RU was recorded (Figure 2A), which represents almost 100% of the calculated maximum binding. In other experiments the binding

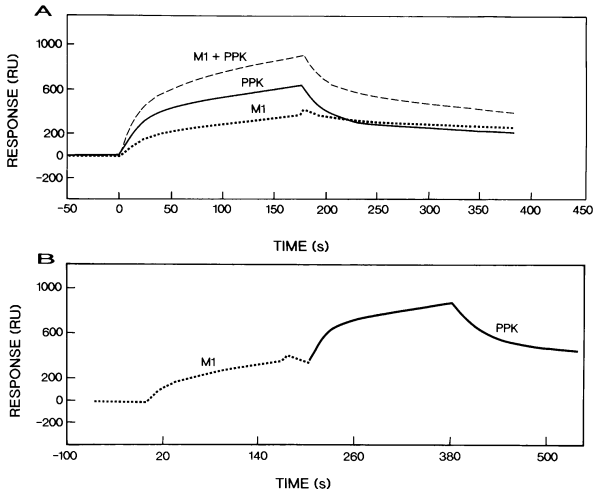

Figure 2 Analysis of the relation between the plasma prekallikrein and M1 protein interactions with HK

(**A**) M1 protein (30 μl, 50 μg/ml) and plasma prekallikrein (PPK; 30 μl, 10 μg/ml) were applied to a sensor chip coupled with HK. Furthermore, a 30 μl sample containing both proteins (50 μg/ml M1 protein and 10 μg/ml plasma prekallikrein) was assayed. (**B**) Samples were also applied as above, except that plasma kallikrein was added following complex formation between M1 protein and HK. Dissociation of bound proteins was measured (expressed in resonance units, RU) following injection of buffer alone.

of plasma prekallikrein to preformed complexes between HK and M1 protein was studied (Figure 2B). The results of these experiments also suggest that M1 protein and plasma prekallikrein can bind simultaneously to HK. Finally, this is also supported by the fact that plasma prekallikrein in an indirect ELISA [17] was absorbed by HK bound to immobilized M1 protein (results not shown).

HK is cleaved at the surface of *S. pyogenes*

Using antibodies against the light chain of HK in Western blot experiments, we analysed the HK bound to and eluted from M-protein-expressing bacteria. Intact HK has a molecular mass of 121 kDa, and the release of the BK nonapeptide from HK results in the formation a two-chain molecule with a heavy and a light chain (63 kDa and 58 kDa respectively) connected by a disulphide bond. The light chain is in the C-terminal part of HK, and as shown in Figure 3 the antibodies against this chain bind to two bands corresponding to the light chain of 58 kDa, and a 45 kDa fragment of the light chain. No band is seen at the place for HK (121 kDa), demonstrating an efficient cleavage of HK at the bacterial surface. These results suggest that HK is released as a consequence of the binding of HK to the streptococci, as HK in plasma occurs only in its intact form [18]. Moreover, HK in plasma incubated with the M-protein-negative *S. pyogenes* strain AP74 [4] showed no degradation, a result that was not affected by the presence or absence of proteinase inhibitors in the plasma sample. The stepwise proteolysis of HK, leading to BK release, starts with the generation of a heavy chain in which the BK peptide is still attached at the C-terminal end. Secondary cleavage by activated plasma prekallikrein then releases the BK peptide. When activated plasma prekallikrein was added in excess to the plasma proteins bound to the streptococci, BK was released and quantified. The level of BK release from strains AP1 and AP46 were 10.7 ± 2.7 and 19.8 ± 7.9 pmol/10^{12} bacteria respectively (values are the means of three experiments $\pm$ 1 S.D.). However, in parallel experiments where activated prekallikrein was added

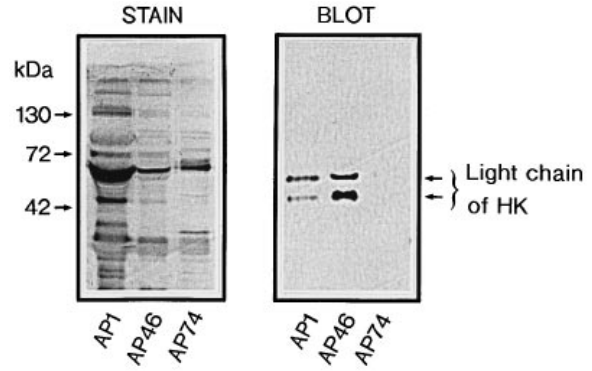

Figure 3 Western blot analysis reveals that HK bound to the streptococcal surface is cleaved

Following incubation with human plasma, bacteria of strains AP1 and AP46, and the M-protein-negative mutant strain AP74, were treated with 30 mM HCl, pH 2.0, to elute plasma proteins bound to the bacteria. The resulting supernatants were subjected to SDS/PAGE (10% gel, w/v) under reducing conditions. Two gels with the same samples were run simultaneously. One was stained with Coomassie Blue ('Stain'), and the other was electroblotted to a PVDF membrane. The membrane was probed with monoclonal antibodies against the light C-terminal chain of HK, followed by peroxidase-labelled secondary antibodies ('Blot'). Molecular-mass markers are shown on the left.

to AP74 bacteria preincubated with human plasma, no release of BK was detected.

DISCUSSION

Fever, oedema and pain are common symptoms in most infectious diseases, and in severe conditions like sepsis, hypovolaemic shock represents a serious and life-threatening complication. Notably, the effects of kinins could explain these symptoms, and it has been suggested that contact-phase activation could be part of the infections process (see [19,20]). In Gram-positive bacteria it was demonstrated that bacterial proteinases can activate the kinin-generating cascade [21,22], and in the case of *S. pyogenes*, an extracellular cysteine proteinase produced by these bacteria was found to cleave kininogens in plasma and release kinins [23]. Moreover, another recent investigation demonstrated that contact-phase factors can be assembled at the surface of certain strains of *Escherichia coli* and *Salmonella* [9] expressing fibrous surface proteins called curli in *E. coli* [24] or thin aggregative fimbriae in *Salmonella* [25].

Our interest in a possible role for the contact-phase system in streptococcal pathogenicity and virulence started with the observation that kininogens, especially HK, showed affinity for streptococcal M-proteins [4]. In HK the binding of M-protein was mapped to the two most C-terminal domains: $D5_H$, which interacts with subendothelial surfaces, and $D6_H$, which forms equimolar complexes with prekallikrein and factor XI. The BK peptide is not included in these domains, and the demonstration here that prekallikrein and M-protein do not compete in their interactions with HK, supports the notion that BK can be released following binding of HK to the streptococcal surface. In this context it is noteworthy that BK release is enhanced when HK is in complex with plasma prekallikrein [26,27]. In the present study it was demonstrated that the binding of HK is followed by its partial cleavage, and that the addition of activated plasma prekallikrein results in a further BK release. The spontaneous cleavage of HK at the bacterial surface indicates that prekallikrein, in its complex with HK, is activated by factor XII. Indeed, preliminary data suggest that both factor XII and factor XI have affinity for streptococcal surfaces. These interactions are currently analysed in detail, as they implicate that activation of the endogenous coagulation pathway may represent another previously unknown virulence mechanism in *S. pyogenes* infections.

From a pathophysiological point of view, a local release of BK, leading to increased vascular permeability and leakage of plasma, could promote spread of the infection and provide nutrients to growing bacteria. As mentioned above, the streptococcal cysteine proteinase efficiently cleaves HK in plasma [23], which, together with the local release of BK at the bacterial surface demonstrated here, could result in a massive BK release. Such a mechanism may help to explain the hyperacute, toxic and often lethal *S. pyogenes* infections that have become an increasing medical problem since the late 1980s [28].

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