Human kininogens interact with M protein, a bacterial surface protein and virulence determinant

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Streptococcus pyogenes, the most significant streptococcal species in clinical medicine, expresses surface proteins with affinity for several human plasma proteins. Here we report that kininogens, the precursors to the vasoactive kinins, bind to the surface of *S. pyogenes*. M protein, a surface molecule and a major virulence factor in these bacteria, occurs in > 80 different serotypes. Among 49 strains of *S. pyogenes*, all of different M serotypes, 41 bound radiolabelled kininogens, whereas 6 M protein-negative mutant strains showed no affinity. M protein of most serotypes bind fibrinogen, and among the 55 strains tested, binding of kininogens was closely correlated to fibrinogen binding ($r = 0.88$, $P < 0.0001$). Western blotting, slot binding and enzyme immunoassay experiments demonstrated that M proteins isolated

from *S. pyogenes* of three different M protein serotypes (M1, M6 and M46) bound kininogens. The affinity between kininogens and M1 protein was determined to be $5 \times 10^7 \text{ M}^{-1}$ and $\leq 10^6 \text{ M}^{-1}$ for high molecular weight (H-kininogen) and low molecular weight kininogen, respectively. The kininogen binding site was tentatively mapped to the N-terminal portion of M1 protein, and this site does not overlap the specific and separate binding sites for albumin, IgG and fibrinogen. Using monoclonal antibodies to, and synthetic peptides of, the kininogen sequence, the major M protein-binding site(s) was mapped to the C-terminal portion of the H-kininogen light chain. We anticipate that the kininogen-M protein interaction contributes to the host-parasite relationship in *S. pyogenes* infections.

INTRODUCTION

Streptococcus pyogenes are pathogenic bacteria in humans responsible for common throat and skin infections. They also cause more rare and severe acute infections, whereas rheumatic heart disease and glomerulonephritis are clinically important sequelae following *S. pyogenes* infections. *S. pyogenes* bacteria express surface proteins with high and specific affinity for several human plasma proteins such as fibrinogen, albumin and Igs. The best-characterized of these surface molecules are the M proteins and the M-like proteins binding Ig (for references see Fischetti, 1989; Boyle, 1990; Kehoe, 1994). M proteins are regarded as major virulence determinants in *S. pyogenes* by conferring on the bacteria the ability to resist phagocytosis, whereas the role for M-like proteins in virulence and pathogenicity is still unclear.

Plasma kininogens, i.e. high molecular weight (H-)kininogen and low molecular weight (L-)kininogen, are multifunctional proteins of complex structure. The two forms of kininogens are generated by alternative splicing of the primary kininogen gene transcript and share their N-terminal domains D1–D4 whereas they differ in their C-terminal domains, D5_H/D6_H and D5_L, respectively (for references see Müller-Esterl et al., 1988). Domains D1–D3 are of cystatin-like structure and inhibit cysteine proteinases such as calpain (D2) and papain (D2, D3). Furthermore, D3 interacts with platelets and endothelial cells and inhibits thrombin-induced platelet activation (Jiang et al., 1992). Domain D4 holds the kinin sequence. The function of D5_L of L-kininogen is still unknown. H-kininogen exposes two unique domains at its C-terminus, i.e. D5_H which binds to subendothelial surface, and D6_H which forms equimolar complexes with prekallikrein and factor XI, respectively. The latter two domains mediate the role of H-kininogen in contact activation (de la Cadena and Colman, 1991).

Several observations support the notion that cysteine proteinase activity is crucial to the biological properties of *S. pyogenes* (Lo et al., 1984; Björck et al., 1989; Kapur et al., 1993). It therefore appeared of interest to investigate a possible interaction between surface proteins of these bacteria and kininogens, the major cysteine proteinase inhibitors of human plasma.

MATERIALS AND METHODS

Bacterial strains and culture conditions

Fifty-five strains of *S. pyogenes* representing 49 different M serotypes and including six M protein-negative mutant strains were obtained from the Institute of Hygiene and Epidemiology, Prague, Czech Republic. The strains have been described (Björck et al., 1984; Åkesson et al., 1990). Strain D471 expressing the M6 protein was from the Rockefeller University (Hollingshead et al., 1986). Streptococci, grown in Todd-Hewitt broth (Difco) at 37 °C for 16 h, were harvested by centrifugation (2000 g, 20 min), washed twice in 0.12 M NaCl/0.067 M phosphate, pH 7.2 (PBS) containing 0.02% (w/v) Na₃Na₂PO₄ (PBSA), and resuspended in PBSA to 2×10^{10} cells/ml.

M proteins and M1 protein fragments

M1 protein from the culture supernatant of strain AP1 (Schmidt and Wadström, 1990) was isolated as described (Åkesson et al., 1994). Pepsin fragments of M1 protein were generated and purified according to Beachey et al. (1978) with modifications as follows. Bacterial cells (2×10^{10}) were resuspended in 2 ml of 0.05 M phosphate buffer, pH 5.8. Pepsin was added to a final concentration of 0.05 mg/ml of bacterial suspension, followed by incubation at 37 °C for 1 h. The incubation was stopped by

Abbreviations used: H-kininogen, high molecular weight kininogen; L-kininogen, low molecular weight kininogen; PBSA, PBS containing 0.02% Na₃Na₂PO₄; PBSAT, PBSA containing 0.05% Tween 20; PVDF, poly(vinylidene difluoride); NHS, N-hydroxysuccinimide.

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Figure 1 Relative locations of the antibody epitopes and of the synthetic peptides in the H-kininogen sequence

The hypothetical domain structure of human H-kininogen is outlined in the centre of the figure. Top: enlargement of domains D5_H/D6_H aligning the target epitopes of various monoclonal antibodies to the H-kininogen light chain (HKL series; Kaufmann et al., 1993). Bottom: enlargement of domain D5_H listing the synthetic peptides (HKHIS series; this work). The relative positions of the segments within the mature H-kininogen molecule are given below the bars (Kellermann et al., 1986). The amino acid sequences of the peptides are (single-letter code) GKEQGHTRRHWDWGHEKQRK (HKHIS-1); HNLGHGHKHERDQGHGHQRG (HKHIS-2); HGLGHGHEQQHGLGLGHGHK (HKHIS-3); FKLDDLEHQQGHVLDHGHK (HKHIS-4); HKHGHGHGHKHNKGKNGKH (HKHIS-5).

adjusting the pH to 8.5 with 7.5% (w/v) NaHCO₃. Cells were sedimented by centrifugation at 10000 *g* for 30 min. The supernatants from five identical extractions were pooled and incubated with fibrinogen-Sepharose for 3 h at 20 °C. The gel was washed extensively with PBS and eluted with 3 M KSCN. The eluents were dialysed against 0.01 M sodium acetate buffer, pH 5.0, and subjected to f.p.l.c. on a HR 5/5 Mono-Q column (Pharmacia f.p.l.c. system) which was eluted with a 40 ml linear gradient of NaCl (0 M–0.5 M) in 10 mM sodium acetate buffer, pH 5.0, at a flow rate of 1 ml/min. Peak fractions were analysed by SDS/PAGE and the relation to M1 protein was determined by amino acid sequencing (Moravek et al., 1986; Åkesson et al., 1994). Recombinant M1 protein fragments were prepared as described (Åkesson et al., 1994). M6 and M46 proteins were expressed in *Escherichia coli* and purified from periplasmic lysates (Berge and Sjöbring, 1993).

Kininogens and antibodies to kininogens

Kininogens were purified from human plasma as previously detailed (Müller-Esterl et al., 1988). Peptides derived from the kininogen sequence were synthesized on a MilliGen 9050 automated continuous flow peptide synthesizer using Pepsyn KA resins (Millipore, Bedford, MA, U.S.A.) and *N*(9-fluorenyl)-methoxycarbonyl chemistry (Meienhofer et al., 1979). The amino acid sequences of the peptides and their relative positions within the amino acid sequence of human H-kininogen (Kellermann et al., 1986) are given in Figure 1. Monoclonal antibodies to human kininogens were raised in mice (Haasemann et al., 1991; Kaufmann et al., 1993). The relative positions of the target epitopes of monoclonal antibodies to domains D5_H and D6_H along with the positional arrangements of the synthetic peptides of D5_H are presented in Figure 1. Polyclonal antisera against H-kininogen were raised in sheep and antibodies (I-107) directed to the common portions of H- and L-kininogen were isolated by extensive immunoadsorption of the antiserum on L-kininogen-Sepharose, followed by the isolation of specific antibodies (I-108) to the unique portions of the H-kininogen light chain (D5_H, D6_H)

by positive immunoselection on H-kininogen-Sepharose (Müller-Esterl et al., 1988; Vogel et al., 1990).

Other proteins and labelling of proteins

Recombinant protein H was purified as described (Åkesson et al., 1990). Papain, pepsin, trypsin and mutanolysin were from Sigma Chemical (St. Louis, MO, U.S.A.). Fibrinogen, polyclonal human IgG and human serum albumin were from Kabi (Stockholm, Sweden) and gelatin from Bio-Rad Laboratories (Richmond, CA, U.S.A.). N-terminal amino acid sequences of the relevant proteins were determined by an Applied Biosystems 477A protein sequencer. Amino acid sequence alignments were performed using the program from Genepro (Riverside Scientific Enterprises, Bainbridge Island, WA, U.S.A.). Proteins were labelled with ¹²⁵I using the Bolton and Hunter (1973) reagent (Amersham Corp., U.K.). H-kininogen was biotinylated as detailed earlier (Hock et al., 1990).

Binding of radiolabelled proteins to bacteria

Bacteria were washed in PBSA containing 0.05% (w/v) of Tween-20 (PBSAT) and the cell concentration was adjusted to 1% w/v corresponding to a total of 2 × 10⁹ cells per ml. Bacteria were diluted as indicated and 200 µl of cell suspension was incubated with radiolabelled protein in 25 µl PBSAT (~ 10⁴ c.p.m.) at room temperature for 30 min. A 2.0 ml volume of PBSAT was added and the bacteria were centrifuged for 15 min at 1800 *g*. The radioactivity of the cell pellet was measured in a γ-counter and expressed as a percentage of the total amount of ¹²⁵I-labelled protein added to the cells. Inhibition experiments were performed with excess amounts of unlabelled proteins (Björck et al., 1984). To digest bacterial surface proteins before the addition of radiolabelled protein, bacteria were treated with mutanolysin, papain, pepsin or trypsin as described (Björck, 1988) followed by testing for binding activity (see above).

Affinity chromatography

CNBr-activated Sepharose 4B, *N*-hydroxysuccinimide (NHS)-activated Sepharose HP, HiTrap-gels and prepacked 1 ml columns were purchased from Pharmacia (Uppsala, Sweden). Coupling of proteins to the gels was performed according to the manufacturer's instructions. ¹²⁵I-labelled H-kininogen was applied to M1 protein- or glycine-HiTrap columns. The columns were extensively washed with PBSAT. Bound material was eluted with 3 M KSCN and the radioactivity of the fractions was measured in a γ-counter.

Competitive binding assay and affinity constants

Constant amounts of M1 protein coupled to NHS-activated Sepharose HP (Pharmacia), and ¹²⁵I-labelled H-kininogen were incubated with varying amounts of unlabelled H-kininogen or L-kininogen. All reagents were diluted in incubation buffer, 0.155 M NaCl, 0.01 M Tris/HCl, pH 7.5, containing 0.25% (w/v) of gelatin and 0.05% (v/v) of Tween-20. Samples were incubated at room temperature for 4 h under gentle shaking. The gel was washed twice with incubation buffer and spun down. The radioactivity of the resulting pellet was measured. Affinity constants were calculated as described (Åkerström and Björck, 1989) using the formula of Scatchard (1949).

Electrophoresis and blotting

SDS/PAGE was performed as described by Neville (1971), using a total concentration of 10 % (w/v) of polyacrylamide and 3.3 % (w/v) of cross-linker. Samples were boiled for 3 min in the sample buffer containing 2 % (w/v) of SDS and 5 % (v/v) of 2-mercaptoethanol. Molecular weight markers were from Sigma. Gels were stained with Coomassie Blue. After electrophoresis the proteins were transferred to poly(vinylidene difluoride) (PVDF) membranes (Immobilon, Millipore) by electroblotting from gels as described by Towbin et al. (1979), using a Multiphor II semi-dry blotting apparatus (Pharmacia). Proteins were also directly applied to PVDF membranes using a slot-blot apparatus from Schleicher and Schuell (Dassel, Germany). The membranes were blocked, washed and incubated with 125 I-labelled proteins as described (Åkesson et al., 1990). Autoradiography was done at -70°C using Kodak X-Omat S films and Cronex Xtra Plus intensifying screens.

E.I.I.s.a.

The indirect e.i.i.s.a. was performed as detailed earlier (Vogel et al., 1990) with minor modifications published elsewhere (Herwald et al., 1993). Briefly, titre plates (Nunc) were coated with serial dilutions (2^n) of the relevant antigen (M1 protein, prekallikrein, α_2 -HS glycoprotein; $4\text{ }\mu\text{g/ml}$), probed with serial dilutions (2^n) of biotinylated H-kininogen (starting concentration $0.5\text{ }\mu\text{g/ml}$; Hock et al., 1990), and followed by the preformed biotin-avidin-peroxidase complex ($2\text{ }\mu\text{g/ml}$) and diammonium 2,2-azino-bis(3-ethyl-2,3-dihydrobenzothiazoline)-6-sulphonate/ H_2O_2 . For the competitive mode, serial dilutions of the competitor (protein, antibody, peptide; starting concentrations $10\text{ }\mu\text{g/ml}$ – $25\text{ }\mu\text{g/ml}$) were mixed with an equal volume of biotinylated H-kininogen ($0.5\text{ }\mu\text{g/ml}$), and the resultant mixture applied to the precoated titre plate as above.

RESULTS

Binding of radiolabelled kininogens and fibrinogen to *S. pyogenes*

Forty-nine strains of different M serotypes were tested for binding of 125 I-labelled H-kininogen, L-kininogen and fibrinogen. Figure 2 shows the results obtained with one H-kininogen preparation and fibrinogen. The majority of the strains (41 out of 49) expressing M protein bound H-kininogen (5%–59% of the total activity was taken up by 2×10^9 cells/ml) above the background (defined as $\leq 5\%$ uptake). Three additional preparations of H-kininogen gave the same binding pattern when tested against the strains of Figure 2, but maximum binding varied between 36% and 60%. The binding of 125 I-H-kininogen was completely blocked in the presence of a molar excess of unlabelled H-kininogen but not of albumin, fibrinogen or IgG (not shown). Because of scarcity of H-kininogen, the reverse experiments were not performed. Binding experiments with 125 I-labelled L-kininogen showed the same pattern as for H-kininogen, but the uptake was consistently lower (never above 25% at 2×10^9 cells/ml). As shown in Figure 2, the 49 strains were also tested for binding of 125 I-fibrinogen and the results were compared with those obtained with H-kininogen (Figure 3). With a few exceptions (strains M38 and M57 are the most noteworthy examples) the two binding activities correlate well ($r = 0.88$, $P < 0.001$), suggesting that similar or even overlapping surface structures are responsible for the interactions. Fibrinogen binds to M protein, a major surface molecule of *S. pyogenes*. We therefore tested 6 M protein-negative mutants (Figure 2, strains AP72–77) which showed low affinity for both 125 I-labelled H-kininogen and fibrinogen. Be-

Figure 2 Binding of 125 I-labelled H-kininogen and fibrinogen to *S. pyogenes*

Fifty-five strains representing 49 different M serotypes and including six M protein-negative mutant strains (AP72–AP77) were used. Binding activities are the mean of three experiments.

Figure 3 Correlation between kininogen and fibrinogen binding of strains of *S. pyogenes*

Figure 4 Effect of various enzymes on the binding of H-kininogen to *S. pyogenes*

Bacteria of the M1 serotype were preincubated in buffer alone ($\square$) or in buffer containing 50 $\mu\text{g}/\text{ml}$ papain ($\circ$), trypsin ($\bullet$), pepsin ($\blacksquare$) or mutanolysin ($\blacktriangle$) in a 10% (2×10^{10} cells/ml) bacterial suspension. Dilutions of bacterial suspensions (starting from 2×10^9 cells/ml) were incubated with ^{125}I -labelled H-kininogen (1×10^4 c.p.m.) for 30 min at room temperature. Cells were washed, centrifuged and the radioactivity of the pellets measured and expressed as a percentage of the total amount of ^{125}I -labelled H-kininogen added to the cells.

Figure 5 Slot binding analysis of the interaction between H-kininogen and surface proteins of *S. pyogenes*

Various amounts of *E. coli*-produced M1 protein, protein H, M6 and M46 proteins were applied to a PVDF filter. The filter was blocked, washed and incubated with ^{125}I -labelled H-kininogen (2×10^5 c.p.m./ml) for 3 h and developed for 3 days; the corresponding autoradiogram is shown here.

Figure 6 Western-blot analysis of the interaction between L-kininogen and M protein

M protein of serotypes 1, 6 and 46, and protein H were run on 10% SDS/PAGE under reducing conditions. After electrophoresis one gel was stained with Coomassie Blue (a), and one gel was blotted onto a PVDF filter which was probed with ^{125}I -labelled L-kininogen (b) for 3 h and autoradiographed for 5 days.

cause M protein is extremely sensitive to trypsin and papain but more resistant to pepsin digestion, we pretreated bacteria of the M1 serotype (strain AP1) with each of the enzymes, and tested for the binding of H-kininogen at different dilutions of the bacteria. For comparison, we included pretreatment with mutanolysin, a bacteriolytic agent which destroys streptococcal cell walls. Uptake of H-kininogen was found to be extremely sensitive to trypsin and papain treatment, whereas preincubation with pepsin and mutanolysin was much less efficient in removing H-kininogen binding activity from AP1 bacteria (Figure 4). However, at higher concentrations pepsin cleaves M proteins in their N-terminal regions (Beachey et al., 1978) and mutanolysin lyses the cells. In summary, the experiments with intact bacteria

Figure 7 Affinity chromatography of H-kininogen on M1 protein-Sepharose

Radiolabelled H-kininogen (4×10^5 c.p.m. in 0.5 ml) was applied to an M1-Sepharose column (a), followed by extensive washing with the application buffer and elution with 3 M KSCN (total recovery was 35% of the applied radioactivity). For comparison the same experiment was performed on a column of glycine-Sepharose (b). In this case only background levels of radioactivity ($\leq 5\%$) were recovered.

Figure 8 Mapping of the H-kininogen binding region of M1 protein

(a) A schematic representation of M1 protein is shown. Ss is the signal peptide, A the N-terminal domain of the mature protein, B1 and B2 are identical repeats followed by a non-repeated IgG-binding domain (S) and three homologous albumin-binding C domains. The D region, finally, includes the putative wall-spanning and membrane-anchoring sequences (Åkesson et al., 1994). M1 protein fragments are indicated. Various amounts of M1 protein and M1 protein fragments (A-C3, S-C3 and Pep1) were applied to a PVDF filter which was incubated with ¹²⁵I-labelled H-kininogen (2×10^5 c.p.m./ml) for 3 h and autoradiographed for 3 days. The resulting autoradiogram is shown in (b).

point to a role of M protein in binding kininogens at the streptococcal surface.

Interaction between purified M protein and kininogens

Genes encoding M protein of serotypes 1, 6 and 46 were expressed in *E. coli*, and the corresponding proteins were purified. M protein of the M1 serotype was also purified from growth medium of *S. pyogenes* strain AP1 (Åkesson et al., 1994). Varying amounts (1 μg–10 μg) of the M proteins were directly applied to PVDF membranes and incubated with a solution containing ¹²⁵I-labelled H-kininogen (Figure 5). The radio-labelled H-kininogen bound to the various M protein preparations in a concentration-dependent manner. For control we applied protein H, the major IgG-binding surface protein of AP1 which shares structural similarity with M protein in the C-terminal but not in the N-terminal regions (Gomi et al., 1990; Åkesson et al., 1994). Unlike M protein, protein H did not interact with kininogens (Figure 5). Similar results were obtained with ¹²⁵I-labelled L-kininogen (not shown). To evaluate further the specificity of the kininogen–M protein interaction, we performed Western blot experiments with proteins M1, M6 M46 (50 kDa–60 kDa) and protein H (42 kDa), respectively (Figure 6a). Application of ¹²⁵I-labelled L-kininogen as the probe yielded positive staining for the various M proteins but not for protein H, thus further underlining the specificity of the interaction (Figure 6b). Similar results were obtained with radio-labelled H-kininogen which gave even stronger signals than L-kininogen (not shown). To further address these differential binding-affinities we immobilized M proteins on Sepharose and applied ¹²⁵I-labelled H-kininogen (4×10^5 c.p.m./ml). A fraction

of 30%–40% of the applied H-kininogen was specifically eluted from the M1 protein–Sepharose (Figure 7a), whereas little if any radioactivity was retained by a control matrix, glycine–Sepharose, under otherwise identical conditions (Figure 7b). Application of the same concentration of ¹²⁵I-labelled L-kininogen revealed about 10% retainment (not shown) suggesting that both types of kininogens bind to immobilized M protein though H-kininogen with a higher affinity than L-kininogen. This conclusion was further substantiated by direct binding studies in solution, demonstrating that the apparent association constant (K_a) for the complex of H-kininogen and M1 protein is 5.0×10^7 M⁻¹, whereas that for L-kininogen and M1 protein is lower by at least a factor of 50 ($\leq 10^6$ M⁻¹). For this reason we chose the high-affinity binding component, H-kininogen, for the subsequent mapping studies.

Mapping of the kininogen-binding region of M1 protein

Our initial results indicated that the binding of kininogen to M protein is not impaired in the presence of albumin, fibrinogen or IgG (cf. Binding of radiolabelled kininogens and fibrinogen to *S. pyogenes* section). Furthermore, we have been unable to block the binding of ¹²⁵I-labelled H-kininogen to Sepharose-bound M1 protein by a large molar excess of fibrinogen (not shown), suggesting that the kininogen-binding site of M protein does not overlap the binding sites for these other plasma proteins. A more direct approach to map the kininogen-binding region of M protein was taken using fragments covering the major domains of M1 protein (a map of M1 protein is presented in Figure 8a). The M1 fragment lacking domain D (A-C3) still binds kininogen and so does a fragment (Pep1) truncated both at the N- and the C-terminus of M1. By contrast, a fragment (S-C3) covering

Figure 9 Indirect (a) and competitive e.i.s.a. (b and c) to probe for the complex-formation between H-kininogen and M1 protein

(a) Titre plates were coated with M1 protein (O), prekallikrein from human plasma (Δ) or α_2 -HS glycoprotein ($\square$). Serial dilutions (2^n) of biotinylated H-kininogen were prepared (starting concentration 0.5 $\mu\text{g/ml}$) and applied to the wells. Bound kininogen was probed by the biotin-avidin-peroxidase complex (2 $\mu\text{g/ml}$) followed by the substrate solution. (b) Plates were coated with M1 protein (4 $\mu\text{g/ml}$). Serial dilutions of antibody I-107 (O), antibody I-108 (Δ), H-kininogen ($\bullet$), L-kininogen ($\blacktriangle$) or α_2 -HS glycoprotein ($\square$), starting from an initial concentration of 50 $\mu\text{g/ml}$ throughout, were mixed with an equivolume (100 μl) of biotinylated H-kininogen (0.5 $\mu\text{g/ml}$), applied to the wells, and bound kininogen probed as above. (c) The set-up of panel B was used except that serial dilutions of peptides HKHIS-1 (O), HKHIS-5 (Δ), or H-kininogen ($\bullet$) were applied (initial concentration 10 $\mu\text{g/ml}$ throughout). Peptides HKHIS-2 to HKHIS-4 were also tested (not shown), but the results were indistinguishable from those for HKHIS-1.

domains S and C but devoid of domains A, B and D loses its binding activity for kininogen (Figure 8). Thus domains D, C1–C3 and S of M1 protein are dispensable for kininogen-binding, which is also true for the N-terminal 24 amino acid residues of domain A. Therefore the remaining part of the A domain and/or the B domains (residues 25–153 of mature M1 protein devoid of the signal sequence) seem to expose the essential structural elements for kininogen binding, i.e. the same region of the M1 protein responsible for fibrinogen binding (Åkesson et al., 1994). Because fibrinogen does not interfere with kininogen binding it appears that the region interacting with kininogen, neighbours, but does not overlap, the fibrinogen-binding site(s) of M protein.

Interference of antibodies with complex formation

To address the corresponding M protein-binding site of the kininogens we set up an e.i.s.a. system where M1 protein was immobilized to a titre plate, and biotinylated H-kininogen in the incubation buffer served as the reporter protein. Figure 9(a) demonstrates that this set-up works efficiently for the H-kininogen–M1 protein interaction. The resultant binding curve is similar to that obtained with the 'natural' complex partner of H-kininogen from human plasma, prekallikrein ($K_a = 8.3 \times 10^7 \text{ M}^{-1}$; cf. Hock et al., 1990), whereas an unrelated protein, α_2 -HS

Figure 10 Interference of monoclonal antibodies with H-kininogen–M1 protein complex formation

A competitive e.i.s.a. was performed where M1 protein (4 $\mu\text{g/ml}$) was the coating protein, 13 monoclonal antibodies directed against distinct epitopes of the human H-kininogen were the competitors (50 $\mu\text{g/ml}$) and biotinylated H-kininogen (0.5 $\mu\text{g/ml}$) was the probe. Bound H-kininogen was visualized by the biotin-avidin-peroxidase technique. The y-axis gives the relative binding (percent of kininogen binding in the absence of a competitor). The x-axis label groups the antibodies according to their target domains in H-kininogen.

glycoprotein (human fetuin), does not recover the biotinylated H-kininogen from the incubation mixture, thus stressing the specificity of the test system. Using a competitive variant of the same e.i.s.a. system we searched for the interference of polyclonal antibodies to the common heavy chain of H- and L-kininogen (I-107) or to the unique light chain H-kininogen (I-108) with the complex-formation between biotinylated H-kininogen and M1 protein. We applied unlabelled H-kininogen and L-kininogen as positive controls and α_2 -HS glycoprotein as an unspecific competitor. Unlabelled H-kininogen was effective in displacing the biotinylated probe whereas L-kininogen was much less efficient and α_2 -HS glycoprotein failed to compete at all (Figure 9b). Antibodies to the unique light-chain portion of H-kininogen were almost as effective as unlabelled H-kininogen while antibodies to the heavy-chain portion shared by the two types of kininogens were poor effectors. These data are consistent with our previous conclusion that H-kininogen binds more avidly to M1 protein than L-kininogen and that the predominant though not exclusive determinants mediating this specific interaction are harboured by the unique light-chain portion of H-kininogen.

Mapping of the M protein binding site in kininogens

To further analyse the relative contributions of the kininogen domains to M protein binding we applied domain-directed monoclonal antibodies recognizing 13 distinct epitopes located on the six domains (D1–D6_H) constituting H-kininogen. None of the antibodies directed to the Ca^{2+} -binding domain (D1), the inhibitor domains (D2, D3) or the kinin-binding domain (D4) interfered, whereas antibodies to the surface-binding domain (D5_H) or to the factor-binding domain (D6_H) were effective (Figure 10). Note that the monoclonal antibodies reduced H-kininogen binding by a maximum of 50% (percent of the total binding in the absence of antibody) whereas polyclonal antibodies were much more effective (cf. Figure 9b). Furthermore, not all of the antibodies directed to the light chain domains were effective interceptors (cf. antibodies HKL10 and HKL12 to D5_H). Moreover, monoclonal antibodies to distinct epitopes of the H-kininogen light chain domain D6_H (HKL2: positions 543–554; HKL17: positions 569–595; HKL22: positions 608–626 of the

mature H-kininogen protein; data from Kaufmann et al., 1993) were almost equally effective, suggesting that several segments of the H-kininogen light chain contribute to M protein binding.

Because antibody HKL12 directed to D5_H was most effective in preventing complex-formation between H-kininogen and M protein, we sought to analyse the effects of peptides derived from D5_H on the complex formation. To this end we synthesized five peptides of 18–20 residues in length which almost completely cover domain D5_H (cf. Figure 1) and applied them in the competitive e.l.i.s.a. (Figure 9c). Peptide HKHIS-5 corresponding to the extreme C-terminal portion of D5_H effectively prevented H-kininogen binding to M protein whereas other peptides were without effect. Together the data from the antibody displacement experiments and from the peptide competition studies indicate that distinct sequence segments dissipated over the light chain domains D5_H and D6_H (covering positions 441–626 of the H-kininogen sequence, cf. Figure 1) contribute directly or indirectly to M protein binding. Our data are compatible with the notion that several possibly discontinuous segments of the H-kininogen light chain form the M protein binding region.

DISCUSSION

M proteins are fibrous, hairlike structures on the surface of *S. pyogenes* (Swanson et al., 1969) which contribute to the ability of the bacteria to persist in the infected host. Thus, in the absence of type-specific antibodies to the M protein, *S. pyogenes* resist phagocytosis by polymorphonuclear leucocytes (Lancefield, 1962). The molecular mechanism(s) behind the antiphagocytic effect of M protein is not fully understood, but it has been suggested that binding to M protein of fibrinogen (Whitnack and Beachey, 1985) and/or factor H of the alternative complement pathway (Horstmann et al., 1988) could play a critical role. Apart from factor H and fibrinogen (Kantor, 1965), M protein of many serotypes shows affinity for IgG and albumin (Schmidt and Wadström, 1990; Åkesson et al., 1994). The binding sites for albumin, IgG and fibrinogen, respectively, were recently mapped to different regions in M1 protein (Åkesson et al., 1994). Thus, albumin was found to interact with the so-called C repeats in the C-terminal half, IgG with the S domain, and fibrinogen with the A and/or B domains of M1 protein (cf. Figure 8). Hence the three most abundant proteins of human plasma have distinct binding regions of M1 protein which do not mutually overlap. This is probably due to the elongated structure of M proteins (Phillips et al., 1981) in which the different binding domains are arranged as pearls on a string.

The present work demonstrates that M protein has yet another binding region for the kininogens distinct from that for albumin, IgG or fibrinogen. We have mapped the kininogen-binding region to the N-terminal part of M1 protein. Interestingly, this region shows little sequence similarity among M proteins of different serotypes. Still the vast majority of M serotypes bind kininogens, suggesting that the three-dimensional structure of the binding region is similar in the various M proteins despite their sequence divergence. Our studies indicate that the major binding region for M protein maps to the C-terminal domains of H-kininogen light chain. Finding that L-kininogen also binds to M protein, though with a considerably lower affinity than H-kininogen, points to the possibility that other portions of the kininogens such as the heavy chain could also be involved in M protein binding.

The biological consequences of the various interactions between M protein and extracellular host proteins are still unclear. However, previous work has demonstrated that the binding of plasma proteins to streptococci and staphylococci changes the

physico-chemical surface properties of the microorganisms (Miörner et al., 1980), which should for instance influence their adherence to epithelial cells. Furthermore, binding of several host plasma proteins to the surface of streptococci might 'camouflage' the invading bacteria thus allowing them to escape from the surveilling host immune system. Kininogens are multifunctional proteins and their presence at the streptococcal surface could affect the host-microbe relationship in more specific ways. *S. pyogenes* for example produces a cysteine proteinase which is active within the bacterial cell (Lo et al., 1984), and is also secreted into the culture fluid as a zymogen (Elliott, 1945) which can be activated by reduction and autocatalytic conversion (Liu and Elliott, 1965). This proteinase, which is identical to streptococcal pyrogenic toxin B (Gerlach et al., 1983), has been implicated in essential functions such as bacterial growth (Björck et al., 1989), activation of interleukin-1 β (Kapur et al., 1993) and mediation of toxic and fatal streptococcal infections in humans (Holm et al., 1992) and in mice (Björck et al., 1989). Given the fact that kininogens are potent inhibitors of cell-associated cysteine proteinase such as calpain (Bradford et al., 1993) one might speculate that H-kininogen bound to the bacterial surface can inhibit the bacterial enzyme via D2/D3 and thereby modulate some of the potential effects of the streptococcal cysteine proteinase.

The accumulation of kininogens at the bacterial surface could also result in the local release of kinins, i.e. proinflammatory agents which increase vascular permeability, contract smooth muscles and induce pain. Such a mechanism could contribute to the rash seen in skin infections and to the redness and edematous membranes of the tonsils and pharynx in cases of pharyngitis caused by *S. pyogenes*. Finally, kininogens have affinity for several cell types (for references see de la Cadena and Colman, 1991) like platelets, granulocytes and endothelial cells. Therefore kininogens bound to *S. pyogenes* (primarily via D5_H and D6_H) could mediate cell-cell interactions between the bacterium and host cells (note that the primary anchor site of kininogens for platelets is located on domain D3).

The host-parasite relationship represents a delicate balance between numerous molecular interactions. The present study further emphasizes the complexity of this relationship and provides a starting point for future work aimed at clarifying some of the mechanisms of importance in microbial virulence and pathogenicity.

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REFERENCES

- Åkerström, B. and Björck, L. (1989) *J. Biol. Chem.* **264**, 19740–19746
- Åkesson, P., Cooney, J., Kishimoto, F. and Björck, L. (1990) *Mol. Immunol.* **27**, 523–531
- Åkesson, P., Schmidt, K.-H., Cooney, J. and Björck, L. (1994) *Biochem. J.* **300**, 877–886
- Beachey, E. H., Seyer, J. M. and Kang, A. H. (1978) *Proc. Natl. Acad. Sci. U.S.A.* **75**, 3163–3167
- Berge, A. and Sjöbring, U. (1993) *J. Biol. Chem.* **268**, 25417–25424
- Björck, L. (1988) *J. Immunol.* **140**, 1194–1197
- Björck, L., Åkesson, P., Bohus, M., Trojnar, J., Abrahamsson, M., Olafsson, I. and Grubb, A. (1989) *Nature (London)* **337**, 385–386
- Björck, L., Miörner, H., Kühnemund, O., Krönvall, G. and Sundler, R. (1984) *Scand. J. Immunol.* **20**, 69–79
- Bolton, A. E. and Hunter, W. M. (1973) *Biochem. J.* **133**, 529–539

- Boyle, M. D. P., ed. (1990) *Bacterial Immunoglobulin Binding Proteins*, Academic Press, San Diego
- Bradford, H. N., Jameson, B. A., Adam, A. A., Wassell, R. P. and Colman, R. W. (1993) *J. Biol. Chem.* **268**, 26546–26551
- de la Cadena, R. A. and Colman, R. W. (1991) *Trends Pharmacol. Sci.* **12**, 272–275
- Elliott, S. D. (1945) *J. Exp. Med.* **81**, 573–592
- Fischetti, V. A. (1989) *Clin. Microbiol. Rev.* **2**, 285–314
- Gerlach, D., Knöll, H., Köhler, W., Ozegowski, J.-H. and Hribalova, V. (1983) *Zentralbl. Bakteriol. Parasitenkd. Infektionskrankh. Hyg. Abt. 1, Orig. A* **225**, 221–233
- Gomi, H., Hozumi, T., Hattori, S., Tagawa, C., Kishimoto, F. and Björck, L. (1990) *J. Immunol.* **144**, 4046–4052
- Haasemann, M., Buschko, J., Faussner, A., Roscher, A. A., Hoebeke, J., Burch, R. M. and Müller-Esterl, W. (1991) *J. Immunol.* **147**, 3882–3892
- Herwald, H., Jahnke-Dechent, W., Abd Alla, S., Hock, J., Bouma, B. N. and Müller-Esterl, W. (1993) *J. Biol. Chem.* **268**, 14527–14535
- Hock, J., Vogel, R., Linke, R. P. and Müller-Esterl, W. (1990) *J. Biol. Chem.* **265**, 12005–12011
- Hollingshead, S. K., Fischetti, V. A. and Scott, J. R. (1986) *J. Biol. Chem.* **261**, 1677–1686
- Holm, S. E., Norrby, A., Berholm, A.-M. and Norgren, M. (1992) *J. Infect. Dis.* **166**, 31–37
- Horstmann, R. D., Sievertsen, H. J., Knobloch, J. and Fischetti, V. A. (1988) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 3765–3768
- Jiang, Y., Müller-Esterl, W. and Schmaier, A. H. (1992) *J. Biol. Chem.* **267**, 3712–3717
- Kantor, F. S. (1965) *J. Exp. Med.* **121**, 849–859
- Kapur, V., Malenski, M. W., Li, L. L., Black, R. A. and Musser, J. M. (1993) *Proc. Natl. Acad. Sci. U.S.A.* **90**, 7676–7680
- Kaufmann, J., Haasemann, M., Modrow, S. and Müller-Esterl, W. (1993) *J. Biol. Chem.* **268**, 9079–9091
- Kehoe, M. A. (1994) *New Compr. Biochem.* **27**, 217–261
- Kellermann, J., Lottspeich, F., Henschen, A. and Müller-Esterl, W. (1986) *Eur. J. Biochem.* **154**, 471–478
- Lancefield, R. C. (1962) *J. Immunol.* **89**, 307–313
- Liu, T.-Y. and Elliott, S. D. (1965) *J. Biol. Chem.* **240**, 1138–1142
- Lo, S.-S., Liang, S.-M. and Liu, T.-Y. (1984) *Anal. Biochem.* **136**, 89–92
- Meienhofer, J., Waki, M., Heimer, E. P., Lambros, T. J., Makofske, R. C. and Chang, C. D. (1979) *Int. J. Pept. Protein Res.* **13**, 35–42
- Miörner, H., Myhre, E. B., Björck, L. and Kronvall, G. (1980) *Infect. Immun.* **29**, 879–885
- Moravek, L., Kühnemund, O., Havlicek, J., Kopecky, P. and Pavlik, M. (1986) *FEBS Lett.* **208**, 435–438
- Müller-Esterl, W., Johnson, D., Salvesen, G. and Barrett, A. J. (1988) *Methods Enzymol.* **163**, 240–256
- Neville, D. M., Jr. (1971) *J. Biol. Chem.* **246**, 6328–6334
- Phillips, G. N., Flicker, P. F., Cohen, C., Manjula, B. N. and Fischetti, V. A. (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 4689–4693
- Scatchard, G. (1949) *Ann. N.Y. Acad. Sci.* **51**, 660–672
- Schmidt, K.-H. and Wadström, T. (1990) *Zentralbl. Bakteriol.* **273**, 216–228
- Swanson, J., Hsu, K. C. and Gotschlich, E. C. (1969) *J. Exp. Med.* **130**, 1063–1091
- Towbin, H., Staehelin, T. and Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 4350–4354
- Vogel, R., Kaufmann, J., Chung, D., Kellermann, J. and Müller-Esterl, W. (1990) *J. Biol. Chem.* **265**, 12494–12502
- Whitnack, E. and Beachey, E. H. (1985) *J. Bacteriol.* **164**, 350–358