Multiple binding of type 3 streptococcal M protein to human fibrinogen, albumin and fibronectin

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Abstract: M proteins are major virulence factors of group A streptococci which enable the bacteria to resist phagocytic attack. Their binding capacity for different plasma proteins seems to be one reason for the antiphagocytic activity of M protein. In the present study we demonstrate that M3 protein, isolated from the streptococcal culture supernatant of strain 4/55, and the recombinant form (rM3), purified from an E. coli lysate after cloning in phage A-EMBL3, show a multiple binding to fibrinogen, albumin and fibronectin in Western blot and dot binding assays. Binding of M3 protein to the multifunctional extracellular matrix and plasma protein fibronectin may not only influence phagocytosis but may also contribute to the adherence of these bacteria to endothelial and epithelial cells.

Key words: Streptococcus pyogenes; M protein; Fibronectin binding; Fibrinogen binding; Albumin binding; N-terminal amino acid sequence

Introduction

M proteins are major virulence factors of group A streptococci and have been implicated in the pathogenesis of acute rheumatic fever and other streptococcal autoimmune diseases. They form a family of closely related proteins protruding from the cell surface as α-helical coiled-coil fibrils [8]. M proteins have been shown to protect streptococci against phagocytosis by granulocytes [20]. The mechanism of this protection is not yet understood, but it has been reported that mainly those molecules of the M protein family which can bind to the plasma proteins fibrinogen and albumin are involved in the protection of group A streptococci against phagocytosis by human granulocytes [40] and intracellular killing [38]. While fibrinogen binding seems to be a general property of M proteins of different types and is accepted as a marker for streptococcal M-protein [16], the binding of M protein to other human proteins like complement control factor H [13], β2-microglobulin [4], human serum albumin (HSA) and IgG [28,29] has been demonstrated only with certain M protein types and is not necessarily a common property of all M proteins. While the antiphagocytic activity of M proteins has been well investigated [20], a contribution of these molecules to streptococcal adhesion is controver-
sial. Tylewska et al. [36] reported that M proteins of different types are involved in the adhesion of group A streptococci to epithelial cells. On the other hand, Caparon et al. [5] did not find differences between M+ and M− strains in adherence to epithelial cells, indicating that a separate component is involved.

Fibronectin (Fn) is one of the cellular matrix components which is a target molecule for bacterial adhesion [2]. Fibronectin binding proteins from group A streptococci other than M protein have been cloned [11,33] and sequence comparison of a group A streptococcal fibronectin binding peptide [34] with FnBP from Staphylococcus aureus [30] revealed similarities in the four repeat binding regions.

In this paper we demonstrate that an M protein can bind to fibronectin. Both M3 protein isolated from streptococci (M3) and recombinant M3 protein expressed in E. coli (rM3) showed affinity not only to fibrinogen and human serum albumin but also to fibronectin.

Materials and Methods

Bacterial strains, bacteriophage and culture conditions

Streptococcus pyogenes strain 4/55 (M type 3 derivative of the original Colebrook strain Richards D58) from the W.H.O. Streptococcus Reference Laboratory, Prague, Czechoslovakia, was propagated in normal human blood before cultivation in Todd Hewitt broth and isolation of M3 protein. This strain was also used to establish a gene library in E. coli (rM3) showed affinity not only to fibrinogen and human serum albumin but also to fibronectin.

Human fibrinogen and human polyclonal IgG was from A.B. Kabi, Stockholm, Sweden. IgA was purchased from Serva, Heidelberg, FRG. Vitronectin was purified according to the method of Yatohgo et al. [42].

Anti-M3 protein rabbit antiserum from our diagnostic laboratory was used and adsorbed with an EMBL3 phage lysate of the infected E. coli strain NM538. Anti-fibronectin rabbit antiserum was raised against gel slices containing the 240-kDa peptide of reduced fibronectin cut out after separation in a 5% SDS polyacrylamide gel. A second anti-fibronectin antiserum from goat was purchased from Sigma.

Labelling of proteins and preparation of affinity media

A number of proteins were conjugated with horseradish peroxidase (HRPO) by using the periodate oxidation method [35] and/or with biotin by using biotin N-hydroxysuccinimide ester (Serva) according to Guesdon et al. [10]. The concentration of activated HRPO added was dependent on the molecular mass of the protein to be labelled. Four molecules HRPO per molecule protein were applied to proteins with molecular mass higher than 100 kDa. For smaller proteins the molecular ratio of HRPO: protein was 2:1.

Albumin and fibrinogen were coupled to CNBr-activated Sepharose (Pharmacia, Uppsala, Sweden) according to the manufacturer's instructions. Three mg protein were added to 1 ml Sepharose.

Construction of the Streptococcus pyogenes gene library and screening of recombinant plaques for fibrinogen binding protein

Streptococcal cells were lysed with mutanolysin [27], and chromosomal DNA was isolated, partially digested with Sau3A, and size fractionated according to Maniatis et al. [23]. Ligation of 11 to 7 kb fragments with BamHI-digested lambda arms and in vitro packaging were performed with a commercial kit (Promega) according to the protocol of the manufacturer. The library was plated and amplified in strain NM539, and, for screening, plates up to 500 recombinant plaques were produced from the amplified stock.
library. Plates were overlaid with nitrocellulose membrane (Schleicher and Schüll) to absorb proteins. The filters were then blocked in 5% defatted skimmed milk in PBS, containing 0.01% Tween 20 for 30 min at 22°C. Then blocking solution was changed and HRPO-labelled fibrinogen was added to a final dilution of 1:500. Filters were incubated for a further 2 h at 22°C and then successively washed for 10 min with blocking solution, PBST and substrate buffer (0.01 M sodium acetate, pH 5.0). Filters were developed in substrate buffer containing 0.02% 3-amino-9-ethylcarbazole and 0.01% H₂O₂. Positive plaques were isolated, amplified and the phages stored at 4°C. Eleven phage clones were isolated which showed reaction with both fibrinogen and with anti-M3 rabbit antiserum. The most stable clone, M3-λ13, was used to isolate M3 protein.

Purification of M protein

Purification from streptococcal culture supernate. Proteins in the culture supernate were precipitated with ammonium sulfate at 70% saturation. The precipitate was dialysed in the presence of 5 mM phenylmethylsulphonyl fluoride (PMSF) against 10 mM ammonium hydrogen carbonate. This crude preparation was applied to a column containing 50 ml fibrinogen-Sepharose. The column was washed with 0.05 M Tris, 0.15 M NaCl buffer, pH 7.5 and bound proteins were eluted with 2 M KSCN buffer. Fractions which were positive in dot binding assays on nitrocellulose with fibrinogen and albumin were pooled, dialysed against 50 mM ammonium hydrogen carbonate and applied to a column containing 20 ml albumin-Sepharose. The column was washed as described above. Those protein fractions eluted with 2 M KSCN buffer which showed binding to fibrinogen and albumin were collected and dialysed against 10 mM ammonium hydrogen carbonate. The material was stored at −20°C in aliquots or lyophilized.

Purification from recombinant phage lysate. Four 1 l shaking bottles each with 250 ml LB were inoculated with 500 µl of E. coli strain NM539 infected with the recombinant phage M3-λ13 in SM medium. Bacteria were grown for 8 h at 37°C. Lysis was completed by addition of 1 ml chloroform to each bottle and continued incubation for 1 h at 22°C. Cell debris was then removed by centrifugation and PMSF was added to the supernatant to a final concentration of 5 mM. Recombinant M3 protein (rM3) was then purified by affinity chromatography on fibrinogen and HSA Sepharose as described above. For N-terminal sequencing, M3 and rM3 were finally purified by HPLC chromatography on a Si300 polyol column (0.75 × 70 mm, Serva, Heidelberg, FRG) in 0.05 M Tris, pH 7.5 containing 1% SDS. Fractions with affinity to fibrinogen and albumin were extensively dialysed against 0.5% acetic acid, 10% ethanol and lyophilized.

SDS-PAGE, Western blot and dot blot analysis

SDS-PAGE was performed according to Laemmli [19]. Proteins were stained with Coomassie brilliant blue. Electrophoretic transfer was performed by the semi-dry method [18]. Immobilon membranes (PSQ, Millipore) were used for amino acid sequencing (see below), and nitrocellulose membranes (Schleicher and Schüll) were used for Western blotting and dot blot assay. The nitrocellulose was blocked with 5% defatted skimmed milk. For the direct binding assay, the membranes were incubated for 1 h with horseradish peroxidase-labelled proteins and successively washed with skimmed milk, PBST and finally with 0.1 M sodium acetate buffer, pH 5.0. The enzyme reaction was visualized with 3-amino-9-ethylcarbazole. If rabbit antiserum were used for detection of antigen-antibody reactions, the blots were incubated in a second step with peroxidase-labelled staphylococcal protein A or, in the case of goat sera, with peroxidase-labelled protein G.

N-terminal amino acid sequencing

Sequencing was performed with lyophilized samples as well as with SDS-electrophoretic separated M3 and rM3 transferred onto immobilon membrane.

Sequences were determined by Edman degradation on Applied Biosystems gas-liquid phase sequencers 470A and 473A according to the man-
ufacturer's instructions. PTH amino acids were identified by online reversed phase HPLC.

Inhibition of fibronectin binding to streptococci

Washed streptococcal cells, grown as described above, were adjusted to 10^9 cells/ml and 100 μl of serial dilutions were applied to nitrocellulose membrane with a dot blot apparatus. Membranes were air dried and then blocked as described above. Control strips of membrane-fixed streptococci were incubated for 30 min with 2 μg Fn or biotin-labelled Fn in 1 ml skimmed milk solution. Duplicate strips were incubated for the same time with 2 μg Fn or biotin-labelled Fn mixed with 20 μg M3 protein in 1 ml skimmed milk. The Fn/M3 protein mixtures in a volume of 50 μl skimmed milk were incubated for 1 h before addition to nitrocellulose strips. After washing of the membranes, binding of biotin-labelled Fn was detected with Streptavidin-peroxidase conjugate. Binding of unlabelled Fn was detected with antifibronectin antiserum from goat and, in a final step, with peroxidase-labelled protein G.

Results

Identification of M3 protein

When affinity-purified M3 and rM3 were analyzed by SDS-PAGE, both preparations were not homogeneous. The stain with Coomassie blue revealed a major band of 62 kDa for M3 and of 60 kDa for rM3 (Fig. 1B). However, after electro-transformation to nitrocellulose in the more sensitive Western blot assay, additional minor bands with molecular masses of 115, 53, 36, 25 kDa for M3 and of 110, 52 as well as 40 kDa for rM3 were identified when probed with anti-M3 typing antiserum (Fig. 1C). From these peptides, the major 62-kDa peptide, as well as the minor 53-kDa and 36-kDa bands of M3 showed binding to fibrinogen as also did the main 60-kDa band of rM3 (Fig. 1E). Fibrinogen binding is considered to be a characteristic property of M proteins [16].

The M3 (62 kDa) purified from S. pyogenes culture medium appeared to be larger than the recombinant product rM3 (60 kDa). This could be a result of limited proteolytic degradation during expression in the heterologous host E. coli. However, it seems that this degradation does not predominantly occur in the N-terminal region of the protein, because both the 62 kDa peptide of M3 and the 60 kDa peptide of rM3 revealed the same N-terminal amino acid sequence: Xaa-Ala-Arg-Ser-Val-Asn-Gly-Glu-Phe-Pro-Arg-His.

The high molecular mass bands of 115 (M3) and 110 kDa (rM3) seem to be dimers of the 62 kDa and 60 kDa peptides, respectively. A similar observation was also reported for M1 protein [28].

![Fig. 1. SDS polyacrylamide gel electrophoresis and Western blotting of M3 and rM3, left lane and right lane, respectively, on each strip. The gel was cut and molecular mass standards (A) as well as M3 and rM3 (B) were stained with Coomassie brilliant blue. The remaining part was transferred onto nitrocellulose. Strips were probed with the anti-M3 typing serum (C) or HRPO-labelled proteins human serum albumin (lane D), fibrinogen (lane E), human IgG (lane F) and human fibronectin (lane G). Further blots were incubated with unlabelled fibronectin (10 μg/ml) and subsequently with anti-fibronectin (lane H) or with anti-fibrinogen rabbit antiserum (lane I). Antigen-antibody reaction was detected with HRPO labelled protein A.](image-url)
Sequence analysis was, however, hampered by the presence of an asparaginyl-glycine peptide bond which is converted to a \( \beta \)-aspartyl peptide bond under the conditions of the Edman degradation [1]. Thus sequencing through Asn-Gly was associated with a drastic reduction in the yield of the liberated phenylthiohydantoins and only very few further amino acids could be determined with the same confidence. However, the results showed unequivocally that both preparations had the same N-terminal amino acid sequence and no other sequence was detected. The amino acid sequence is highly homologous to the N-terminus of a partial translated DNA sequence of type M3 strain 3-3/317 [25]. However, in position 4 of the present protein sequence we found a serine instead of a threonine in position 4 of the translated sequence, if the signal peptide of the reported M3 sequence [25] was not considered.

**Binding properties of M3 protein**

In recent binding experiments with a number of human proteins [28,29] we could demonstrate that the 49-kDa peptide of M1 protein expressed binding sites for albumin and the Fc-fragment of IgG besides the known fibrinogen binding. Comparable binding experiments were performed with the proteins M3 and rM3 in this study. As shown by Western blotting (Fig. 1), the main peptides of both M3 (62 kDa) and rM3 (60 kDa) showed a pronounced affinity for peroxidase-labelled albumin and fibrinogen (Fig. 1D, E) but, in contrast to M1 protein [28], not for IgG (Fig. 1F). Albumin binding, especially to the minor bands, was more pronounced than binding of fibrinogen (Fig. 1D, E). However, in a second experiment the affinity-purified preparations M3 and rM3 were applied in dots to nitrocellulose, and binding was

![Fig. 2. Dot binding of M3 and rM3 applied onto nitrocellulose in different concentrations. Binding was assayed with different HRPO-labelled proteins: HSA, human serum albumin; Fbg, human fibrinogen; IgG, human immunoglobulin G; IgA, human immunoglobulin A; Fn, human fibronectin; Vn, human vitronectin; CnI and CnIV, collagen types I and IV. Blots were developed as described in Materials and Methods. Both preparations show binding to HSA, Fbg and Fn.](image-url)
tested with a number of peroxidase-labelled proteins (Fig. 2). In this experiment we found binding to fibrinogen, albumin and also to fibronectin. Because this reaction with peroxidase-labelled Fn did not occur with the denaturated M3 proteins in the Western blot assay, M3 and rM3 were pretreated with SDS sample buffer and heated for 2 min also before the dot blot assay. After this procedure, both M3 and rM3 retained their affinity for albumin and fibrinogen, but lost finding to fibronectin (not illustrated). Thus it seemed that denaturation by SDS destroyed Fn-binding to M3 protein. On the other hand, because peroxidase-labelling could also impair the binding site on the fibronectin molecule, we performed a two-step assay. A parallel strip of the Western blot was incubated with unlabelled Fn, followed by incubation with rabbit anti-fibronectin antiserum, and the reaction was monitored after probing with peroxidase-labelled protein A. In this case, a weak binding was found, indicating that a part of the M3 molecules retained their binding properties after SDS-electrophoresis and Western blotting. Only the main bands of M3 (62 kDa) and rM3 (60 kDa), which we think represent the complete functional M3 protein, were able to bind fibronectin (Fig. 1H). This indicates that unaltered conformations of both fibronectin and M protein are necessary for binding to each other. The reaction was specific because anti-fibrinogen rabbit antiserum (Fig. 1I) and rabbit normal serum (not illustrated) did not give any signal. Moreover, Fn-binding to streptococci of strain 4/55 could be inhibited by addition of M3 protein (Fig. 3). This suggests that, in the case of strain 4/55, the M3 protein is the Fn-binding component exposed on the cell surface. The same result was obtained when biotin-labelled Fn (Fig. 3A, B) or unlabelled Fn (Fig. 3C, D) were used. For detection of cell-bound unlabelled Fn we used anti-Fn antiserum from goat (Fig. 3C, D), because we found that strain 4/55 also expressed an IgG binding protein on the cell surface which was different from M3 and which bound to human and rabbit IgG but not to IgG from goat.

Discussion

In this study we show that M3 protein is a multifunctional streptococcal component which can bind to the human proteins fibrinogen, albumin and fibronectin. While binding of fibrinogen and albumin to M proteins of other types has already been described [9, 28, 29] the binding of M3 to fibronectin shows, for the first time, that an M protein can interact with a protein which is not only present in plasma but also in the extracellular matrix of epithelial cells. In contrast to the interaction with fibrinogen and albumin, the binding of M3 to fibronectin was sensitive to denaturation with SDS, which indicates a conformation-dependent binding. Recently, a multifunctional binding pattern was also described with M1 protein, which showed affinity for fibrinogen, albumin and, in contrast to M3, for IgG and not for fibronectin [28, 29]. This suggests that M proteins of different types can individually express varying affinities for human proteins, whereas fibrinogen binding is considered as a stable property of M proteins.

Adherence, colonisation and subsequent invasion of mucosal surfaces are the first steps of systemic streptococcal infection [2].

The anti-opsonizing role of M protein in phagocytosis, occurring in the body fluids after
invasion of streptococci, is well established [20]. In this step of infection, the binding of the plasma proteins fibrinogen [38,40] and albumin [38] to M-positive streptococci have been reported to interfere with the phagocytosis of streptococci by granulocytes. While fibrinogen prevented ingestion of streptococci by granulocytes [40], albumin binding was found to support intracellular survival and multiplication of ingested streptococci [38].

The present observation that M3 protein can interact with fibronectin indicates that, in some types of group A streptococci, M protein is involved in the adherence to tissue and epithelial cells. Recent studies of Tylewska et al. [36], which also demonstrated an enhanced binding of M positive group A streptococci and M protein to epithelial cells, support an active role of M protein in streptococcal adhesion. In contrast, Capron et al. [5] showed that M+ and M− mutants of type 6 streptococci did not differ in their adherence to epithelial cells. Furthermore, fibronectin binding proteins from streptococci have been cloned [11,33] and a C-terminal binding peptide with a molecular mass of 40 kDa was found to be different from M protein [34]. Another protein, group A streptococcal glyceraldehyde-3-phosphate-dehydrogenase was shown to be a multifunctional protein also with affinity to fibronectin [24].

In conclusion, it is obvious that group A streptococci can express different fibronectin binding proteins. The presence of more than one receptor protein in one bacterial strain is not without precedent, because two different fibronectin binding proteins have already been described for staphylococci [15,30] and for Streptococcus dysgalactiae [21,22].

The nature of Fn binding to group A streptococci was controversially discussed in the past. Lipoteichoic acid was first described to be involved in streptococcal adherence to epithelial cells and in binding to Fn [3,7,39]. However, more recent reports and our results provide evidence that fibronectin binds with a higher affinity to proteins of group A streptococci [6,11,24,31,33]. Thus, the binding of fibronectin to lipoteichoic acid as well as to different streptococcal cell wall proteins indicates a multifunctional adhesion mechanism of group A streptococci to epithelial cells.

Fibronectin is a high molecular mass glycoprotein which has been found in soluble form in body fluids [26] and in insoluble form in the cellular matrix and basement membranes [12]. The role of fibronectin as an important mediator of adherence is well established [26]. It is a multifunctional human protein mediating substrate adhesion of eukaryotic cells through its binding of specific cell surface receptors [14,41]. Because fibronectin binds to several species of bacteria, including staphylococci [17] and streptococci [32], the expression of different bacterial receptors for the same target, fibronectin, can be considered as an evolutionary requirement of these bacteria as human pathogens.

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